SCREENING AND CHARACTERIZATION OF STARCH PRODUCED FROM MARINE MICROALGAE *Klebsormidium flaccidum* GN-2

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SCREENING AND CHARACTERIZATION OF STARCH PRODUCED FROM MARINE MICROALGAE, *Klebsormidium flaccidum* GN-2

by

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ROZI NURAIKA BINTI RAMLI

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

%	Percentage
α	Alpha
ADP-	Adenosine diphosphate-
ATP	Adenosine Triphosphate
$CHCl_3$	Chloroform
$CHCl_3$/MeOH	Chloroform/Methanol
CO_2	Carbon dioxide
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride
$CuSO_4 \cdot 5H_2O$	Copper sulfate pentahydrate
D-	Dextrorotatory
DNA	Deoxyribonucleic acid
Fe	Iron
$FeCl_3 \cdot 6H_2O$	Iron (III) Chloride Hexadhydrate
g	Centrifugal force
g/g	gram per gram
g/L	gram per Liter
H_2O	Water
H_3BO_3	Boric acid
ha	hectare
HCl	Hydrochloric acid
ΔH_{gel}	Enthalphy of gelatinization
K	Potassium
Mg	Magnesium
mg/L	Milligram per litre

ml/min	Milliltre per minute
mg/mL	Milligram per millilitre
MOPS	3-morpholinopropane-1-sulfonic acid
$MnCl_2 \cdot 4H_2O$	Manganese (II) Chloride tetrahydrate
Na_2-EDTA	Disodium ethylenediaminetetraacetate dihydrate
$NaH_2PO4 \cdot 2H_2O$	Sodium dihydrogen phosphate dihydrate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
$Na_2SiO_3 \cdot 5H_2O$	Sodium silicate pentahydrate
$(NH4)_6Mo_7O_{24} \cdot 4H_2O$	Ammonium heptamolybdate tertahydrate
N2	dinitrogen
P	Phosphorus
PHGH	Polyhexamethylene guanidine hydrochloride
Pi	Orthophosphate
PPi	pyrophospahte
PVOH	Polyvinyl alcohol
R^2	Coefficient of determination
rRNA	Ribosomal ribonucleic acid
TFA	Trifluoroacetic acid
ton	Tonne
T_o	Onset temperature
T_p	Peak temperature
T_c	Completion temperature
µL	microlitre
µm	Micrometer
v/v	volume per volume
w/v	Weight per volume

w/w	Weight per weight
$ZnCl_2$	Zinc chloride

LIST OF ABBREVIATIONS

AOAC	Association Official Agricultural Chemists
ANOVA	Analysis of Variance
BBM	Bold Basal Medium
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CCBCCS	Centre for Chemical Biology
CCS	Commercial Corn Starch
CEMACS	Central of Marine Coastal Study
C. reinhadrtii	*Chlamydomonas reinhadrtii*
C. sorokiniana	*Chlorella sorokiniana*
C. vulgaris	*Chlorella vulgaris*
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
DTG	Derivative Thermogravimetric
DW	Dry weight
EPA	Eicosapentaenoic acid
FA	Fatty acid
g	gram
h	Hour
GOPOD	Glucose oxidase/peroxidase
kDA	kilodalton

K. flaccidum	*Klebsormidium flaccidum*
L	Litre
L. aromatica	*Limnophila aromatica*
LD	Lipid droplet
M	Molar
m	metre
mg	milligram
min	minute
ml	millilitre
mM	milliMole
MW	Molecular weight
N	Nitrogen
nm	nanometers
N. oculata	*Nannochloropsis oculata*
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
OD	Optical density
PCR	Polymerase Chain Reaction
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
ppt	part per thousand
PUFAs	polyunsaturated fatty acids
rpm	Revolutions per minute
RSM	Response Surface Methodology
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulfate

SEM	Scanning Electron Microscope
sp.	Species
SPSS	Statistical Package for Social Sciences
ST	Stack of thylakoids
TAGs	Triacylglycerols
TEM	Transmission Electron Microscopy
TGA	Thermogravimetric analysis
U.S	United State
USM	Universiti Sains Malaysia
V	Vacuoles
yr	year

LIST OF APPENDICES

SARINGAN DAN PENCIRIAN KANJI YANG DIHASILKAN DARIPADA MIKROALGAE MARIN, *Klebsormidium flaccidum* GN-2.

ABSTRAK

Kanji adalah komponen utama yang terkandung dalam dua per tiga dalam piramid diet manusia dan sepertiga kanji digunakan dalam aplikasi bukan makanan seperti bio-bahan api dan bioplastik. Produk yang dihasilkan dari kanji boleh terbiodegradasi dan membantu mengurangkan pencemaran alam. Walau bagaimanapun, masalah persekitaran seperti penebangan hutan dan kekurangan makanan berasaskan kanji akhirnya akan berlaku apabila permintaan kanji untuk produk bukan makanan meningkat. Peningkatan permintaan untuk bio-bahan api dari tanaman kanji akan menyebabkan persaingan antara makanan dengan bahan bakar. Mikroalga yang mudah tumbuh dan mesra alam telah membuka potensi revolusi baru untuk produk berasaskan kanji. Dua jenis mikroalga marin berjaya disaring dari laut Pulau Pinang dan diasingkan. Berdasarkan penemuan identifikasi morfologi dan molekul, terdapat 2 spesis yang dikenalpasti sebagai *Klebsormidium flaccidum*, GN-2 dan *Nannochloropsis oculata* YG-2. *K. flaccidum* GN-2 menghasilkan 16.84% kanji, biojisim 0.81 g/L dan *N. oculata* YG-2 masing-masing 3.06% dan 0.40 g/L untuk kanji dan biojisim. *K. flaccidum*, GN-2, menghasilkan kanji yang lebih tinggi dipilih untuk pengoptimuman menggunakan Kaedah Sambutan Permukaan (KSP) untuk meningkatkan pengeluaran kanji. Kanji yang dihasilkan ialah 19.06% dibawah keadaan optimal pada kadar kemasinan 35.53 ppt, 23 jam pendedahan cahaya dan kadar aliran 1% v/v karbon dioksida. Pengoptimuman nutrien dikaji berdasarkan penyingkiran nitrogen (N), fosfor (P) dan ferum (Fe) kerana kekurangan nutrisi akan mengubah fisiokimia mikroalga. Namun penurunan biojisim (N = 0,98 g/L, P = 0,22

g/L dan Fe = 0,24 g/L) dan kanji (N = 6,39%, P = 4,62%, Fe = 4,46%) menggambarkan bahawa penyingkiran nutrisi dalam *K. flaccidum*, GN-2 tidak dapat meningkatkan kandungan kanji. Kanji tersebut dicirikan dan dibandingkan dengan kanji jagung komersial untuk dari segi kandungannya. Butiran saiz granul kecil (1µm) dan kandungan amilosa tinggi (25.52%) dalam *K. flaccidum*, GN-2 berbanding kanji jagung komersial (ukuran 7µm, amilosa 23.04%) menjadikan kanji mikroalga dicadangkan sebagai bahan terbaik untuk aplikasi bio-filem yang kuat dan teguh kerana saiz granulnya yang kecil amat diperlukan untuk menghasilkan bio-filem yang berkualiti. Gelatinisasi kanji *K. flaccidum*, GN-2 lebih rendah (ΔHgel = 9.45 J / g) daripada kanji jagung komersial (ΔHgel = 11.63 J/g) disebabkan oleh kandungan amilopektin yang rendah dan entalpi gelatinisasi yang rendah. Hasil retrogradasi dan sineresis menunjukkan kanji mikroalga mengandungi kestabilan yang hampir sama dengan kanji jagung komersial. Kanji mikroalga berupaya bergabung semula untuk membentuk struktur yang lebih teratur dalam 72 jam dan penuaan sepenuhnya selepas kitaran ke-3 seperti kanji jagung komersial. Kesimpulannya, mikroalga marin yang diasingkan dari lautan Pulau Pinang berjaya menghasilkan kanji. Walaupun kanji yang dioptimumkan tidak menunjukkan prestasi peningkatan yang tinggi, namun ciri-ciri yang dikaji terhadap kanji dari *K. flaccidum* GN-2 setanding dengan kanji jagung komersial. Oleh itu, ia boleh menjadi alternatif untuk menghasilkan pelbagai produk lestari yang mengurangkan persaingan dengan kanji daripada sumber tanaman.

SCREENING AND CHARACTERIZATION OF STARCH PRODUCED FROM MARINE MICROALGAE *Klebsormidium flaccidum* GN-2

ABSTRACT

Starch is the main component that belongs to two third of the human diet pyramid and another one third of starch is used in non-food applications such as biofuel and bioplastic. Products produced from starch are biodegradable and help in reducing the environment pollution. However, environmental problem such as deforestation and shortage of food will eventually happen if the demands of starch from non-food and food products increase. Simultaneously, crops using as feedstocks for biofuel will result in food versus fuel implication. To resolve this problem, the attentions of researchers have been driven towards microalgae. The easy growing and environmentally friendly microalgae has opened a new potential of revolution for starch-based product. In this research two strains of marine microalgae have been successfully isolated and screened from Penang Sea. Based on morphological and molecular identification, these 2 strains are designated as *Klebsormidium flaccidum* GN-2 and *Nannochloropsis oculata* YG-2. *K. flaccidum* GN-2 produced 16.84% of starch and 0.81 g/L biomass while, *N. oculata* YG-2 produced 3.06% and 0.40 g/L ofstarch and biomass, respectively. Microalgae *K. flaccidum* GN-2 was chosen for optimization using Response Surface Methodology (RSM) to increase production of starch since it yield higher starch than *Nannochloropsis oculata* YG-2. The starch produced was 19.06% under the optimized conditions of 35.53 ppt salinity, 23 hours of light exposure and flow rate of 1 v/v of carbon dioxide. The nutrient optimization was studied based on depletion of nitrogen (N), phosphorus (P) and iron (Fe) as the

nutrient depletion will change the physiochemical of the microalgae. Decreasing of biomass (N = 0.98 g/L, P = 0.22 g/L and Fe = 0.24 g/L) and starch (N = 6.39%, P = 4.62%, Fe = 4.46%) however illustrated that depletion of the nutrients in *K. flaccidum* GN-2 could not increase the starch content. The starch was characterized and compared with commercial corn starch (CCS) for its properties. Small granule (1 µm) and high amylose content (25.52%) in *K. flaccidum* GN-2 were compared to commercial corn starch (size 7 µm, amylose 23.04%) suggested that starch from *K. flaccidum* GN-2 as the best potential feedstock for strong and stiffer films due to small size of granule. Gelatinization of starch from *K. flaccidum* GN-2 is lower (ΔHgel = 9.45 J/g) than commercial corn starch (ΔHgel = 11.63 J/g). This is due to the lower amylopectin content in *K. flaccidum* GN-2 leads to lower crystallinity and less energy needed to dissociate the molecule within the granule. The retrogradation and syneresis results showed that microalgae starch contains similar stability with CCS as the gelatinized starch able to reassociate to form more ordered structure within 72 hours and completely ageing after 3rd cycle like CCS. In conclusion, marine microalgae isolated from Penang Sea was successfully produced starch. Even though the optimized starch did not show significant increased, however the characteristics studied in starch from *K. flaccidum* GN-2 is comparable to the CCS starch. Hence it could be an alternative to produce various sustainable products that least compete with crops starch.

CHAPTER 1

INTRODUCTION

1.1 Research background

Starch is a natural carbohydrate storage polymer accumulated in plants (Sangwan *et al.,* 2014). It is a completely biodegradable polysaccharide and one of the most abundant renewable resources. It has been considered as an excellent candidate to partially substitute synthetic polymer in packaging due to its abundance, biodegradability, and low cost. This polysaccharide consists of two D-glucose polymers: amylose and amylopectin (Perezs *et al.,* 2010). The usage of starch in daily life is well-known as in food and non-food product. The sources of starch are mainly crops such as potato, corn, and tapioca. Gifuni *et al.* (2017) wrote in the study, starch from corn, maize and wheat are allocated two-thirds used in the food industries and one third as non-food products.

The sustainable awareness had hit the market as now starch crops had become the feedstock for the green product such as bioethanol and bioplastic (Harun *et al.*, 2010). United State (U.S) (2009) is the top producer for bioethanol production using corn as feedstock and it constitutes 50.7% of the total world market while Brazil as the second producer contribute 40.2% production in the green energy market by using sugar cane as a main source. These two sources (corn and sugarcane) are renewable in nature and available in plenty (Rahman & Miller, 2017). Recently the bioplastic production increased from approximately 2 million tons in 2014 to almost 6.7 million tons in 2018 which was mostly derived from corn and potatoes (Rahman & Miller, 2017). However, using foods such as corn and potatoes as feedstocks for biopastic production leads to increase food prices, which damages the ethical well being of entire

human population. In 2020, Asia produced 46% of bioplastic in international market and had become the largest producer followed by Europe, 26%, North America, 17%, South America, 10% and Australia 1% (European, Bioplastic, 2020). The total land used to grow crops for bioplastic production is reported as 0.7 million hectar and which is expected to increase up to 1.1 million hectar in the next five years as reported in European Bioplastic (2020). These yet accounted for land used for agriculture area, biofuel, food and feed. In 2005, the U.S produced 18.9 billion L of bioethanol from 20% of the total corn land which was counted only 1% of the total fuel consumed in the U.S per year (Harun *et al.*, 2010). The demand on these crops will be increased along with the land usage. Furthermore, it will lead to shortage of starch supplied and deforestation is needed to fill more demand of the crops. Also, the starch producing crops take long period to grow before harvesting. Though the product is a renewable product, however the process to produce these crops are not sustainable for a long-term.

The properties of starch mainly depend upon the climatic conditions, which differ with the cultivation region. The component of starch inside the plant plays a big role in product formation. Hence the starch is characterized by analyzing its properties such as the granule size, amylose/amylopectin ratio, turbidity and solubility and swelling power (Gifuni *et al.*, 2017).

Microalgae is an innovative alternative as the excellent candidates for starch accumulation and production. Microalgae are widely undergoing research for its high carbohydrate content. The application on bioethanol is now expanded due to its content and the main reason for turning to microalgae is because it is a renewable source. Microalgae pose advantages of high biomass yield and able to grow in wide range

environment conditions (Maheswari &. Ahilandeswari, 2011). Besides, it can grow faster, easy and it is able to absorb more carbon dioxide and greenhouse emitting gases for photosynthesis (Giuliano *et al.,* 2011). Thus, microalgae can be harvested within a short period as compared to plants and crops and hence can meet the increasing demand for feedstock (Harun *et al.,* 2010). The other prominent features of microalgae are it has simple growth requirement, can be easily grown in various aquatic environments such as freshwater, saline water or municipal wastewater and in a controlled condition which will lead to consistency of cell produce without affected by the outer environment conditions (Shilton, J.,2009; Shilton *et al*., 2008).

Starch contents in microalgae species is based on cultivation conditions and cultivation time. Both of marine microalgae and fresh water are in interest since freshwater species could sequester CO_2 from power station on inland and marine species could sequester CO_2 from power station located along seashore. Mostly, freshwater microalgae strains have been studied for starch accumulation and less attention paid to marine microalgae. Generally, total carbohydrate content in microalgae is about 20% dry weight (DW) and starch content is about 10% DW (Lauren *et al.,* 2012). The low-cost yet high yield from microalgae had reported by Brányiková *et al.* (2011) using freshwater alga *Chlorella vulgaris* which able to produce almost 48% of starch content since it accumulates large amounts of carbohydrates through photosynthesis and stores as starch in cells. Freshwater microalgae, such as *Chlorella, Chlamydomonas, Scenedesmus, Spirulina* (Chisti 2007; Gouveia and Oliveira 2009; Gouveia *et al.,* 2009; Lopes da Silva *et al.,* 2009; Miao & Wu 2006; Morowvat *et al.*, 2010; Rodolfi *et al.,* 2009; Xu *et al.,* 2006) contain large amounts (50%) of starch and glycogen (Ueda *et al.,* 1996) which have been studied intensively for starch and lipid production. *Chlorella vulgaris* was significantly high

in lipid, up to 70% of lipid accumulation when cultivated in high-salinity condition. *Dunaliella* sp. contains up to 21.2% of lipid content (Gong & Bassi, 2016; Singh *et al.,* 2016). Most of marine microalgae strains are highly robust species that maintains high growth rates at extreme pH, temperature and salt concentration such as *Dunaliella tertiolecta* and *Klebsormidium* sp. (Katz I *et al*., 2009). Tolerance in high salt concentration enable these marine microalgae to be cultivated outdoor at high salt concentration thus minimizes the contamination compared to fresh water or soil microalgae. Marine microalgae, *Dunaliella tertiolecta* accumulates starch nearly 60% and its special feature i.e. lack of cell wall enabling easy cell lysis which lower the cost of processing (Ike *et al.,* 1997; Pick & Avida, 2017). Whereas *Chlorella obliquus*, *Tetraselmis subcordiformis*, consist almost 35% of starch content (Hon-Nami, 2006; Ji *et al.*, 2011; Zheng *et al.*, 2011). Matsumoto *et al.,* (2003) had screened green marine microalgae, NKG 120701 which content high carbohydrate (53%) for bioethanol production and used marine bacteria, *Pseudoalteronomas undina* as enzyme catalyser. *Chlamydomonas reinhardtii* had been studied by cultivated mixotrophically or heterotrophically by adding organic compounds (acetate) for starch accumulation nearly 58% of dry weight (Heifetz *et al.*, 2000). However, it is not an ideal target strain for starch production due to the diminished carbon dioxide (CO_2) fixation ability and higher risks of contamination. Most of the marine microalgae studied were done for bio-ethanol production but neither for starch production or for its characterization. Therefore, there is a need to carry out intensive research on marine microalgae for high starch productivity and consequently use as feedstock for as an alternative of current starch sources.

1.2 Research scope and objectives

Overall, this research focuses on isolation of new marine species of microalgae from Penang Sea to optimize and extract the starch. The samplings were done at Penang Sea at various places. The growth profile and the starch inside the cell were studied. The isolated marine microalgae then used to optimize the culture conditions for higher starch production. The culture conditions were categorized as physical (salinity, light, and carbon dioxide) and nutrients (nitrogen, phosphate and iron) factors which influenced the starch production. Then, the starches obtained under different culture conditions were characterized and compared with commercial corn starch (CCS) in order to measure the capability in applying into a product. Amylose and amylopectin ratio, size of the starch granule, swelling power and solubility, gelatinization and also retrogradation were analyzed. The objectives of this study were:

i. To screen and isolate high starch producer of marine microalgae from selected sea area in Penang Island
ii. To optimize the factors (salinity, light duration and carbon dioxide) affecting high starch production using response surface methodology (RSM).
iii. To extract and characterize the starch from marine microalgae, *K. flaccidum* GN-2 and comparison with CCS.

CHAPTER 2

LITERATURE REVIEW

2.1 Starch

Polymeric carbohydrate or starch is a biopolymer produced by photosynthesis process. It is one of the major carbons and energy storage compound produced in green plants and algae (Halley *et al*., 2007). Starch is widely available insoluble biopolymer which is completely biodegradable in nature. It has significant values in human life as it serves as main carbohydrate source in the human diet (Pfister & Zeeman, 2016) and acts as a staple food (Karmakar *et al.,* 2013). Starch is claimed as safe since, the carbon in plants is derived from atmospheric carbon dioxide (CO_2), hence the degradation of starch either by biodegradable or incineration will result in no net gain in CO_2 (Laycock *et al*., 2014). Most of the starches in their native states are intolerant towards the broad range of processing conditions and poor functional properties **(**LeCorre & Angellier-Coussy, 2014). Hence, characteristics of starches are important in order to modify it to functional properties.

2.1.1 Molecular structure of starch

Starch is a complex polysaccharide made up of a large number of glucose units joined together by glycosidic bonds. It consists of amylose and amylopectin (Table 2.1). Amylose and amylopectin exist as a component of discrete, semi-crystalline aggregate and amorphous concentric layers in starch granule (Svihus *et al.,* 2005). These polymers are solely α-D-glucose connected by α-1-4-linkage glycosidic bond which forms into shorter or longer chains. Amylose is a lesser component of the granule with 20%-30% constituents of the granule. Amylose chains are joined by α-1-4-linkage non-branched

molecule (Figure 2.1a) responsible for the amorphous regions of the starch granule. The structure of helix containing hydrogen atoms gives the hydrophobic properties and this causes amylose to form a type of clathrate complex with free fatty acids, fatty acid components of glycerides, some alcohols and iodine (Pérez *et al*., 2009). Amylose contributes to the gel formation and pastes when the starch is being cooked. This resulted from the retrogradation or reassociation of solubilized starch polymers after being heated and linear polymer amylose contribute highly for these properties (Thomas & Atwell, 1999). Amylose consists of higher degree of polymerization (DP) than amylopectin, about 1500 to 6000 DP as it only contains a linear chain.

Amylopectin constitutes 70%-80% of the starch granules hence responsible for the granule nature of starch crystalline lamella (cluster structure). Other than connected by 1,4 linkage bonds for linear chains, amylopectin also connected by α-1,6 bonds for branched chains (Figure 2.1b) (Hathwaik & Cushman, 2017; Halley *et al*., 2007). The linkages estimated about 4-6% linkage within an average amylopectin molecule (Hood, 1982).

Table 2.1 Characteristics of amylose and amylopectin (Thomas &Atwell, 1999).

Characteristics	**Amylose**	**Amylopectin**
Shape	Linear	Branched
Linkage	α-1,4	α-1,4 & α-1,6
Molecular weight	<0.5 million	50-500 million
Films	Strong	Weak
Gel formation	Firm	Non-gelling to soft
Colour with iodine	Blue	Reddish-brown

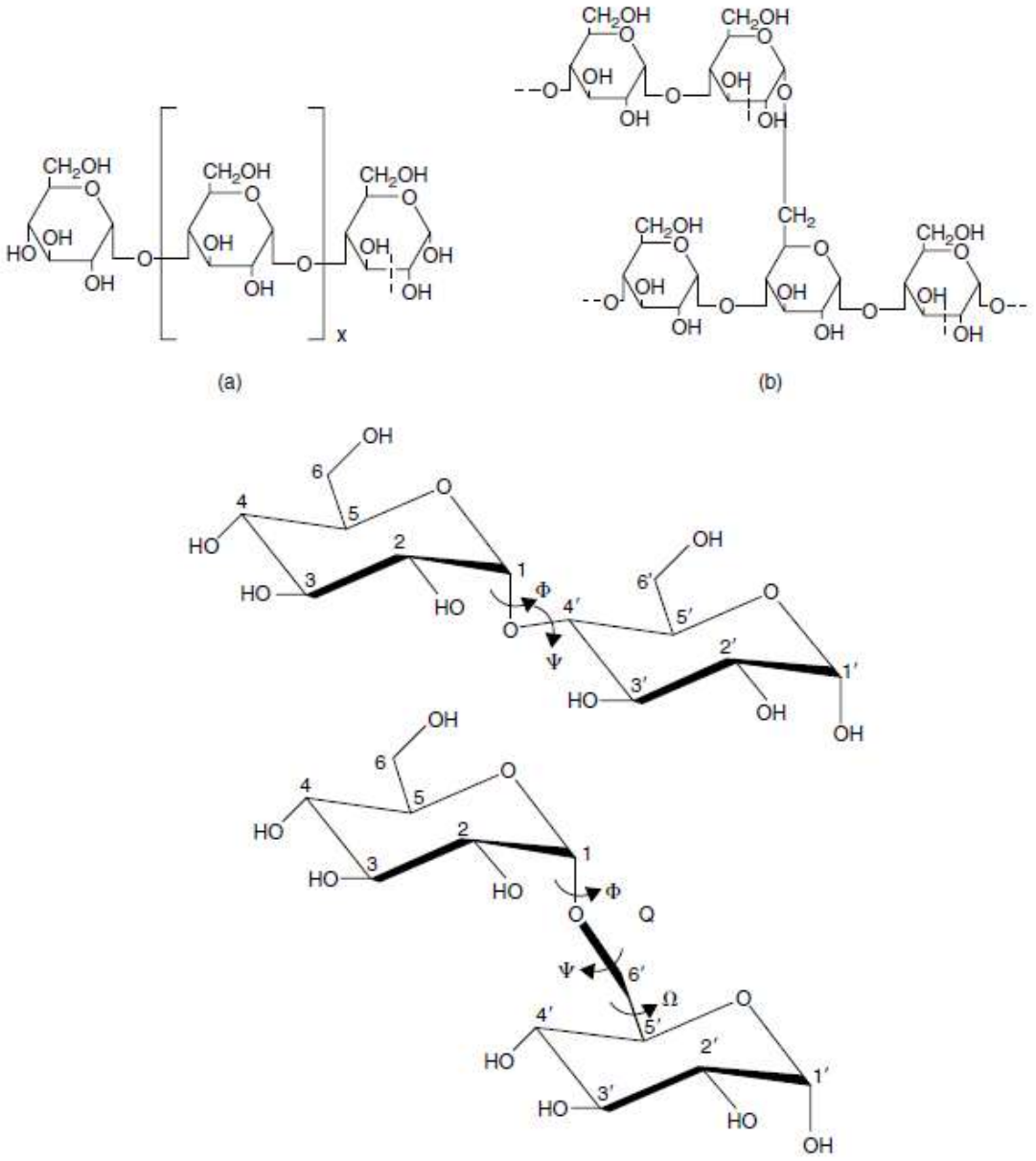

Figure 2.1 Schematic diagram of (a) amylose and (b) amylopectin with a branch point at O6 position. (c) Schematic representation of the disaccharide components of starch: α-1,4 and α-1,6 with the torsion angles that define the conformations at the glycosidic linkage between two contiguous residues (Pérez *et al*., 2009).

This small percentage has resulted in more than 20,000 branches in an average molecule of starch studies show that there are two types of chains in amylopectin, which are small and large chains. The small chain posed an average degree of polymerization

(DP), around 15. Whereas the large chain is about 45 DP. DP is the number of α-1,4 linkage bond in the starch chain that contribute to the crystalline nature of amylopectin and an ordered arrangement of the amylopectin molecules (Robin *et al.*, 1974; Hizukuri, 1986). The branch existed in the amylopectin molecule makes the properties different from amylose. The properties such as retrogradation and gelatinization are different as the structure affects the reaction. For example, starch paste that contains all amylopectin is a waxy starch and possess non-gelling and a gummy texture (Thomas & Atwell, 1997).

Amylose and amylopectin are arranged in an organized order on native starch granule. The arrangement is less ordered when heating as the bond holding the molecules is disrupted. The disordered reaction varies depending on the starch content for amylose and amylopectin. When the starch granules are heated in water, the native granules swell until their structure disintegrates and amylose and amylopectin are released in aqueous suspension. The constituent's ratio of amylose and amylopectin in starch granule is important for the starch application and it varies with different starch sources (Table 2.2). These two molecules affect the functionality of the product based on the starch granule, gelatinization, retrogradation and texture.

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Table 2.2 Amylose and amylopectin content in common food starches (Thomass & Atwell, 1999).

Starch type	Source	Amylose content (%)	Amylopectin content (%)	Diameter (μm)
Dent corn	Cereal	5	75	5-30
Waxy corn	Cereal	<1	>99	5-30
Tapioca	Root	17	83	4-35
Potato	Tuber	20	80	5-100
High-amylose corn	Cereal	55-70 (or higher)	30-45 (or lower)	5-30
Wheat	Cereal	25	75	1-45
Rice	Cereal	19	81	1-3

2.1.2 Granular structure of starch

Other than amylose and amylopectin; protein, lipid, moisture and ash are also present in starch granules (Tester *et al*., 2004). Protein in starch granule has been divided into two types, surface and integral starch granule. The integral proteins are embedded and bound in the structure of amylose and amylopectin while, surface proteins are loosely associated with the exterior of the starch granule. Lipid in starch granule contains phosphate groups and similar in structure of emulsifier lethicin with

one fatty acid group per molecule (lysophospholipids). It able to form a complex structure with amylose wherein the fatty acid group is aligned in the core of amylose helix. This formed complex but very stable structure and can only dissociate at high temperature. The quantities of each property are starch source dependent such as in tuber and root starches (potato and tapioca) contain less protein and lipid than cereal starches (dent corn). Potato contains 0.1% of protein and 0.1% of lipid whereas dent corn contains 0.35% and 0.80% of protein and lipid respectively. These components will affect the gelatinization and flavor profile of the starch. Lower content oflipid and protein in potato gives bland flavor to the starch. Moisture constituet is 12% in starch powder ash typically less than 0.5% in dry biomass. Ash is accumulated from mineral elements and inorganic salt. Its content varies depending upon the sources of the raw material, agronomic practices, milling procedures and type of chemical modifications made on the starch. The chemical composition in starch granules is related to the starch granules surface, hardness (Finnie *et al*., 2010) and the crystallinity (Ao & Jane, 2007)

2.2 Characteristics of starch

2.2.1 Granule morphology and size

Starch granule comes in a regular and irregular shape. The size, shape and structure of the granules vary depending on the sources (Table 2.2). The size exists in large A-, medium B- and small C- type. The diameter of granule varies depending on the sources, commonly from less than 1 µm to 100 µm. The shape exists in regular and irregular such as in spherical, ovoid and angular (Singh *et al*., 2010). Some small granules of starch, such as found in rice and oats cohesively bound together in an organized manner and these are known as compound starch granules. Starch from rice 3-9 µm, wheat 3-

40 and potato is among the biggest size of starch granule, 15-80 µm (Table 2.3, Figure 2.2). Microalgae *C. sorokiniana* shows lower in size granule than rice, 0.8-5.3 µm (Gifuni *et al.,* 2017). This characteristic is responsible for their physiochemical and functional characteristics such as gelatinization, crystallinity, and solubility (Wijaya *et al*., 2019) as it will involve the process chosen to yield the product and also what type of product suit best for the size (Singh *et al*., 2010).

Table 2.3 Characteristic of starch sources (Gifuni *et al.,* 2017; Kevin Bael, 2017).

Characteristics	**Rice**	**Wheat**	**Corn**	**Tapioca**	**Potato**	***C.sorokiniana***
Size (µm)	3-9	3-40	15-25	20-35	15-80	0.8-5.3
Shape	Oval and polygonal	Oval	Hexagonal	Hexagonal	Oval	Oval and sphere
Color	Very white	Greyish white	Yellowish white	Greyish white	White	Greenish white
Taste	Neutral	Cereal taste	Protein taste	Light off-taste	Potato taste	-
Gel structure	Soft and creamy	Firm	Firm	Sticky	Sticky	Firm

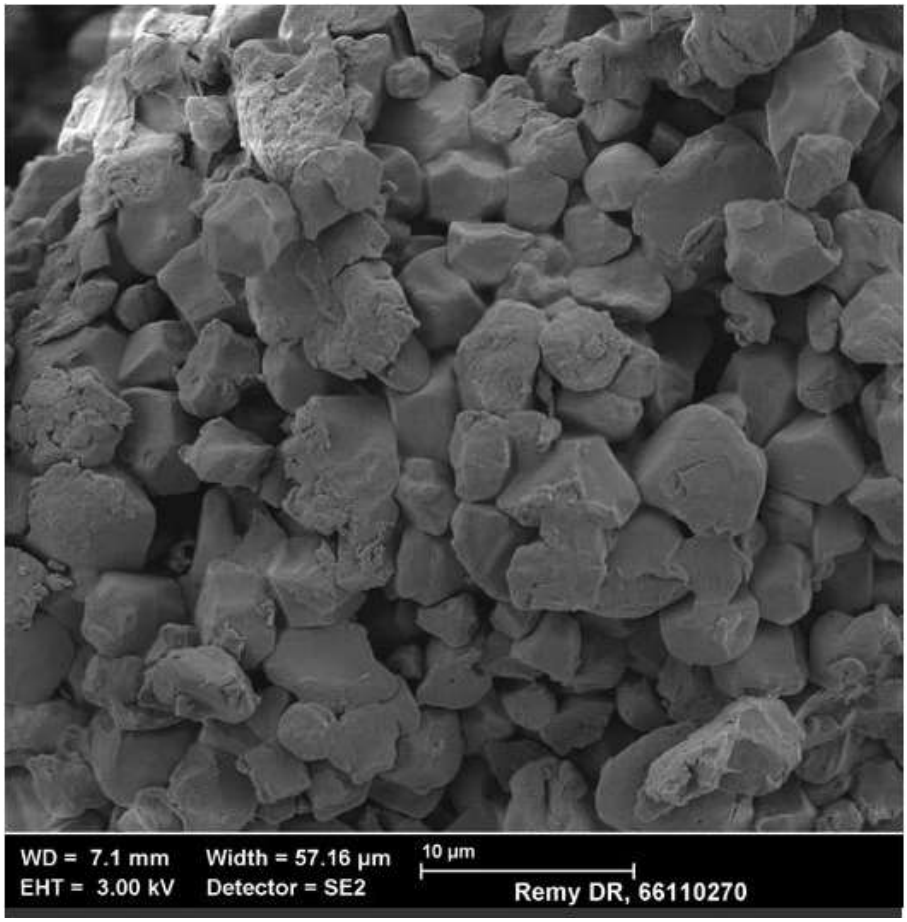

Figure 2.2 Scanning electron microscope (SEM) of regular rice starch (Kevin Bael, 2017).

A high specific surface area on small size of starch granule has become main interest in industries. This property increases the quality of the starch-based product. The high specific surface area increases the viscosity; hence more crosslinking reaction occurs between the granule molecules. This enhances starch modification that provide better surface adhesion, high gelatinization temperature and resistance of the granules.

These small granules would work best as a fat replacer in free-fat food formulation, carrier material in pharmaceutical and cosmetic and also high quality of biodegradable film (Lindeboom *et al*., 2014). This interest commonly found in rice, oats, certain species microalgae range 0.5-3 µm (Gifuni *et al*., 2017; Wilhelm *et al*., 1999). However,

the separation process between big and small granule is quite expensive that will be resulted in a limitation for commercialization.

2.2.2 Amylose and amylopectin ratio

Amylose and amylopectin ratio are studied to explain the starch physicochemical and functional features. Just like starch size and morphology, the starch functionality is highly affected by amylose and amylopectin ratio and it varies in different sources (Table 2.4). Amylose indicates the properties such as water binding capacity and thickening, whereas amylopectin in charge on the crystalline network for a stable double helix structure (Singh *et al.*, 2010; Yoo & Jane, 2002). High amylose content would lead to strong and stiffer thermoplastic film with the help of amylopectin that forms a translucent paste that remains fluid when cooled. Amylopectin with longer chain forms a stable crystalline structure (Shewry *et al*., 2009). Shorter chains of amylopectin forms an unstable double helix structure which letting the structure easily to disrupt even at a low temperature of heat. However, the ratio will also depend on the product wanted as low amylose content starch (12%-16%) suitable to be applied in thicker and binder. high amylose content (18% - 25.3%) will form a stronger and stiffer film (Gifuni *et al*., 2017, Hassan *et al*., 2013).

Table 2.4 Comparison between the starch source for amylose content (Gifuni *et* al., 2017; Wijaya *et al*., 2019)

Biomass	**Amylose (%)**
Banana	Not available
Chestnut from Yanquan	35.10
Wheat (*Triticum timopheevii*)	28.1-33.8
Cassava	19.5
Corn	22.4-32.5
Rice	7.83-18.86
Potato	25.17
L.aromatica	23.78
C. sorokiniana	17

2.2.3 Swelling power and solubility

Swelling power is indicated as the ability of water holds the starch granules when heated in water. Solubility is the percentage of the amount of starch leached out into the supernatant in the swelling volume determination. The major factor that controls these characteristics is the strength and character of the micellar network within the granule which depend on the degree and interaction between the molecules in the starch granule (Teli *et al*., 2009). When the starch is heated in water, hot water is being absorbed into the granule molecules and causes the granule to swell. Hydrogen bonds between water hydroxyl groups are disrupted that triggers solubilization. Then amylose leached out from granule and granule absorbs water which causes the granules to swell. Swelling power is inversely proportional to solubility. The main factors that influence swelling power are amylose and amylopectin ratio, degree of polymerization (DP), length of amylose and amylopectin, and also the molecular weight (Hoover, 2001). Starch sample

with low amylose content and largergranule size have high swelling power due to the amylopectin interaction between structures. Whereas the solubility is temperature-dependent hence, the higher the temperature, the higher the solubility will be. Rice with amylose content 7.83% posses 26.06 g/g of swelling power at 95°C and solubility 0.29% at the same temperature. However, corn with high amylose content, 22.4% shows low swelling power, 22% at 95°C and 22% solubility at the same temperature (Wijaya *et al*., 2019).

2.2.4 Thermal properties

Thermal properties of starch granules are analyzed to assess the thermal processability of the starch in the industry especially in food and plastic industries (Tananuwong & Reid, 2004; Tufvesson *et al.*, 2003; Yu & Christie, 2001). There are two types of thermal properties, gelatinization and mass loss. Gelatinization is the breakdown of starch granule from an ordered granular structure into a disordered state in water when raw starch granules are heated in water leads to the breakdown of the semi-crystalline and molecular structure dispersing the starch polymer in solution (Ratnayake & Jackson, 2009). Differential Scanning Calorimetry (DSC) is widely used to measure the heat of gelatinization of starch indicate a net sum of all endothermic processes that take place during heating. It shows the entire phase transition process by rapid direct scan from room temperature to a certain above-boiling temperature accurately. The advantage of using DSC, it permits precise control of the sample temperature while keeping the sample in a physically closed system (Chinachoti *et al.*, 1990). There are three stages in the starch gelatinization process: first is granular swelling by slow water absorption; the second stage is the rapid loss of birefringence by absorption of large amounts of water by granules; and the last stage is leaching of

the soluble portion of starch into solution, transforming the granules into formless sacs and it is irreversible (Ratnayake & Jackson, 2008). The curve of DSC will emphasize on four points, onset (T_o), peak (T_p), conclusion (T_c) and enthalpy of gelatinization (ΔHgel). ΔHgel has indicated the overall measure of crystallinity on quantity and quality and an indicator of the loss molecular order within the granule (Liu *et al.*, 2006).

Mass loss of starch sample is measure using Thermogravimetric analysis (TGA) technique as a function to temperature or time while the sample is subjected to a controlled condition in controlled atmosphere (Liu *et al*., 2012). The technique widely used method to study on the starch and starch-based materials for its thermal stability and decomposition. Derivative Thermogravimetric (DTG) is the first derivates of TGA is a sensitive measurement and provide the information on the relative rates of volatilization and polymer decomposition (Liu *et al*., 2012**)**. DTG will be useful for starch degradation analysis to differentiate if there are any overlapping mass loss events, shapes identification and maxima of mass loss processes and identify the minor mass loss**.** In the curve, for each of weight loss, there will be a Differential Thermal Analysis (DTA) curve to represent a separate event in a temperature range **(**Prime *et al.*, 2009). Thermal decomposition will be imposed three-stage, first is water loss, second is starch decomposition which will happen around 300°C and the last stage is carbonization reaction, decomposed of other molecules which happen around 500°C (Liu *et al*., 2012**)**.

The thermal analysis is well related to the amylose and amylopectin ratio. Increasing of amylopectin and amylose ratio leads to increasing of the thermal decomposition temperature and sequence of activation energy. This is due to the higher of MW of amylopectin, hence more stable microstructures which more energy needed to breakdown the starch molecules. Amylose polymer affects mainly on the onset and

increasing of amylopectin increases the temperature of chain decomposition since amylopectin responsible for the crystalline of the starch structure (Liu *et al*., 2012).

2.2.5 Turbidity

Turbidity is analysed to characterize the retrogradation behaviour of diluted starch paste. Retrogradation is referred to as disaggregated of amylose and amylopectin chains in a gelatinized starch paste reassociate to form more ordered structures. After the starch granules swelled, the cooled starch molecules will form a new network between amylose and amylopectin chains that was leached out of the granules during gelatinization. The factors that affect the turbidity development are the granule swelling, granule remnants, leached of amylose and amylopectin chain length. The changes in turbidity during storage is due to the interaction between leached amylose and amylopectin chains that lead to the development of function zone (Pereira & Hoover, 1999; Ambigaipalan *et al.*, 2013).

2.2.6 Syneresis

Syneresis is the process where the separation of liquid form a gel or colloidal suspension due to contraction motion (Li, Kim, Huang, Jia, & Xu, 2010). The freeze-thaw method is applied to measure the syneresis of pasted starch to evaluate the stability of cold-resistant starch. After starch gelatinized in the presence of water, the molecule will reassociate during cooling into gel-like structures hence affect the functional and sensory properties of foods. The reorganization happens during cooling storage where the water is being released from the starch molecule. The syneresis is important to evaluate the functional properties for the stability of the products (Huang *et al*., 2008;

Kim *et al.*, 2008). During the freezing storage process, the gelatinized starch separates into starch-rich and starch deficient regions, which leads to the formation of ice crystals consequently, the starch becomes sponge-like structure with increased retrogradation in the starch-rich region (Lorenzo *et la.*, 2009; Zhang & Simsek, 2009). This characteristic is important to understand the stability of the starch for example in prebaked frozen bread, by adding an additive would improve the stability during the freezing process as the water molecules in the additives such as dextrin offers better stability during storage which improve the shelf life of the products (Bárcenas *et al.*, 2003).

2.3 Sources of starch and its synthesis

The starch synthesis starts in chloroplast with triose phosphate as the starting phosphate, which forms fructose-1,6-biphosphate and later be converted to fructose-6-phosphate, glucose-6-phosphate and glucose-1-phosphate by reactions which is catalyzed by fructose-1-6-bisphosphotase, hexose phosphate isomerase and phosphoglucomutase, respectively. The glucose-1-phosphate is converted to ADP-glucose via ADP-glucose pyrophosphorylase in the plastid as reaction that requires ATP and generates pyrophosphate (PPi). PPi is hydrolysed into two orthophosphate (Pi) molecules using a specific inorganic pyrophosphatase (PPi), hence leading the reaction towards ADP-glucose synthesis. Therefore, the glucose moiety of ADP-glucose is transferred to the non-reducing end of the terminal glucose of a growing starch chain (Hathwaik & Cushman, 2017).

There are two types of starch, native and modified starch. Native starch is a pure and naturally occurring form of starch whereas modified starch is the physically or chemically modified (Chung *et al.*, 2009) starch to enhance quality and suitability towards the desired products. Native starch consists of long-chain carbohydrates that

are insoluble in cold water and have different degrees of swelling depending on the type of starch and temperature. Hence, modifications such as annealing, osmotic pressure treatment are applied to the native starch to improve the functional properties (Pukkahuta *et al*., 2007) and resulted as modified starch. Modified starch can be altered according to the need suits in food application, pharmaceutical or other applications. Taro starch (*Colocasia esculenta)* is physically and chemically modified starch by increasing the viscosity, swelling volume and good retrogradation process and the resulted modified starch is used in food thickener and drink diet shake (Karmakar *et al*., 2013). Starch and its derivates are produced from a wide range of sources such as plants, (corn) and algae (microalgae) (Laycock *et al*., 2014).

2.3.1 Starch from plants

Starch commonly found in plants as it is the major polysaccharide. Annual production for primary starch sources was estimated as 46.1 million tons from corn, 9.1 million tons from cassava, 5.15 million tons from wheat and 2.45 million tons of potato (Röper & Elvers, 2008). European countries produce around 60% of starch and Asia produces 20% of starch from wheat which is used in dextrin and modified starches (Bertonili, 2010). The biggest corn starch producer, The United States (U.S) produced 98% of corn starch and became the largest bioethanol producer (Harun *et al*., 2010) and corn starch contributed 70% out of starch produced in the world (Röper & Elvers, 2008). Thailand has become a major producing country for cassava starch, 3.5 $x10^6$ tons, followed by China and Indonesia. However, corn is still the main starch sources in the world because starch from root possesses high viscosity with low retrodegradation which requires formulation in making specific products while corn starch does not require formulation step (Bertonili, 2010).

2.3.2 Starch from algae

Number of living algae estimated varies from 30,000 to more than 1 million species, but most of the reliable estimates refer to the numbers given in AlgaeBase has documented 32,260 species of organisms generally regarded as algae. According to the AlgaeBase, almost 28,500 unidentified species. The total number of algae species arelikely to be about 72,500, out of which more than 20,000 species are diatomic. Algae are photosynthetic organisms and hence produce the same storage compounds as well as use similar defence strategies against predators and parasites like a plant. However, algae do not have roots, stems, leaves, or well-defined vascular tissues, like plants. Algae capture CO_2 and transform it into organic biomass such as lipid and starch. Algae grow into dissimilar forms such as microscopic single cells, macroscopic multicellular loose or filmy conglomerations, matted or branched colonies, or more complex leafy or blade forms, which contrast strongly with uniformity in vascular plants (Barsanti & Gualtieri, 2006).

Macroalgae and microalgae are two types of algae which abundantly found on earth either in water from springs and rivers to hypersaline Ocean, lagoons and salt lakes or land. These two types of algae are rich with their by-product which could be turn into any products like food or non-food products. The ubiquity of these organisms together with the flexibity of their metabolic requirements makes many algal species easily available for investigation, collection, or simple observation. Algae has been utilized by human for over hundreds of years as food, fodder, remedies, and fertilizers.

Macroalgae is a multicellular, macroscopic, eukaryotic and autotrophic organism and normally known as seaweeds. This organism lives freely near the seabed (Suganya *et al*., 2016) and could be found in every wet environment in land, freshwater

or land (Leandro *et al*., 2019). The seaweed appearance resembles non-arboreal terrestrial plants. The energy produced is stored as starch and different polysaccharides of the large molecular chain. It could be found in numerously based on the body (thallus) color which causes by their pigments such as red (Rhodophyta), brown (Ochrophyta) and green (Chlorophyta) macroalgae depending on the group classified. Green algae produce ulvan which contain carotene and xanthophylls and chlorophylls *a* and *b* as pigments. Whereas red algae contain chlorophylls *a* and *d*, carotenoids and phycoerythrin pigment which is responsible for their staining. Brown algae content pigments such as fucoxanthin, chlorophylls *a* and *c* and carotenoids and reserve substances such as oils, and polysaccharides. (Barsanti & Gualtieri, 2014; Leandro *et al*., 2019). Generally, macroalgae do not contain lipid but high in carbohydrate which could be fermented to produce the desired product.

Like plants in terrestrial land, macroalgae with kelps size up to 60 m (Figure 2.3) has become important sources in aquatic ecosystems because it serves as great food sources for animals, and present in the saltwater aquarium as natural filters by reducing the levels of nitrite and phosphate from water. The bioremediation is done by bioabsorption and bioaccumulation (Neveux *et al*., 2018; Yu *et al*., 2016; Henriques *et al*., 2017). Seaweed is used in many countries for various purposes, like industrial phycocolloids extraction or extraction of compounds with antiviral, antibacterial, or antitumor activity (Shannon & Abu-Ghannam, 2016), human nutrition, livestock and biofertilizer (Pereira, 2016). *Laminaria* sp. was identified to has 12% (w/w) of protein content which could be utilized for human consumption however it contains 2% (w/w) lipid but no starchthat is why it could not be used in fuel production and sugar fermentation (Bruton *et al*., 2009). Other than human consumption, it is widely applied as hydro-colloid production. A survey done in 1995 stated that 221 species of seaweed

were collected for human application which 145 species for food, 101 for hydrocolloid extraction (Bruton *et al*., 2009). The substances that synthesize by macroalgae have great potential to be used in wide area such as the pharmaceutical and food industry.

Figure 2.3 (a) Longline with *Laminaria* after 8 months of growth (Yellow Sea, China) (b) Longline showing attached *Laminaria* plants (South Korea). (c) Young sporophytes growing on a longline) (Sahoo & Yarish, 2005).

Microalgae grow by photosynthesis process also produced starch as the metabolite by product. Microalgae or phytoplankton are microscopic unicellular, photosynthetic organism which fond abundantly on earth. The photosynthesis mechanism in microalgae is like land-based plants due to their simple cellular structure. Many microalgae can produce big amounts (8-57%) of starch function as storage materials (Brányikova *et al.,* 2011). Generally, the main components of microalgae are lipids (7-23%), carbohydrates (5-23%), and proteins (6-52%) (Onen Cinar *et al*., 2020).

Other components contain in microalgae are calcium (0.1-3.0%), sulphur (0.4-1.4%), copper (18-102 mg/kg), iron (1395-11,101 mg/kg), manganese (45-454 mg/kg), selenium (0-0.5 mg/kg) and zinc (28-64 mg/kg). The efficiency, fast growth, and large biomass plus environmentally friendly behaviour of microalgae have drawn interest for researchers and manufacture to study more on the starch on the derivative starch product (Harun *et al*., 2010). Gifuni *et al*., (2017) reported 48% of starch content in freshwater microalgae, *Chlorella sorokiniana.* Small starch granules (1 µm) and high gelatinization temperature (110 °C) make it as suitable source in making good quality product such as film packaging and bioplastic (Onen Cinar *et* al., 2020).

2.4 Starch application

Starch has a wide range of applications either in foods or non-food industry. Cheap raw materials and biodegradable has attracted many manufactures to produce a starch-based product such as bioplastics, paper coating, pharmaceutical sector, industrial binder, the textile industry as stiffener the textiles, increase the mechanical strength and resistance to friction wear (Laycock *et al*., 2014). Starch that produced in the world is reported that 57% of the starch is consumed in food industries, and 43% is used in non-food industries (Bertolini, 2009).

In food industries, the main usage of starch is in syrup production and formulation of ready meal and sauces (Bertolini, 2009). In Europe, 30% of produced starch is used in sweet, drink and fruit processing whereas convenience food such as bakery, food ingredients and dairy products contribute 27% of starch produced. The remaining starch is used in the paper industry (28%), chemical and fermentation (14%) and feeds (1%). North America is one of the starch producers from corn, however, 73%

of starch produced is used in the U.S. for the production refinery products such as syrup, maltodextrins and fructose (Röper & Elvers, 2008).

After food, the paper industry is the second largest consumer of starch for almost 10 million tons per year. When the cost for cellulose pulp increases, the manufacturers look for cheaper fillers. Starch could replace as the filler after undergoing some modifications either by oxidation or enzymatic hydrolyzed. Modified starch is also used as paper coating hence improved the strength and printability of the paper which also applied in textile and laundry finishing products (Lawala *et al*., 2005; Van der Maarel *et al*., 2002)

Bioplastic from starch is one of the promising products that naturally decompose. Thermoplastic-starch is currently in high demand when normal plastic (polyethlene) has become an environmental problem for being not decomposing even after 100 years and the incineration of it has led to air pollution as well. The raw materials from corn and potato have become great sources for bioplastic production (Mohammadi Nafchi *et al*., 2013). In the bio-film packaging industry, starch is gelatinized with a polymer such as polyvinyl alcohol (PVOH) or glycerol for the better flexibility and rigidity (Sadeghizadeh-Yazdi *et al.*, 2019) to increase the shelf-life of the fruits and vegetables (Pagella *et al*., 2002). The film can be used as a plasticizer in edible coating and films (Larotonda *et al*., 2004; Cyras *et al.*, 2006).

In pharmaceutical industries, native starch is modified either by physical, chemical or enzymatic reaction to control the drug delivery system. Phosphate-modified starch is used to prepare iron oxide nanoparticles and mixed with mixtoxantrone which improves in drug concentration and targeting toward cancer cells (Alexiou *et al*., 2006). Rice starch is chemically modified to produce metronidazole and has led to a controlled