CO_2 BIOFIXATION AND CARBOHYDRATE BIOSYNTHESIS BY LOCALLY ISOLATED ACIDOPHILIC MICROALGAE FOR BIOBUTANOL PRODUCTION THROUGH SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

®	Represent
™	Trade Mark
°C	Degree Celcius
±	Plus-Minus sign
µ	Micro
%	Percentage

LIST OF ABBREVIATIONS

AGB	1,4-Alpha-Glucan Branching Enzyme
AS	Agar Streaking
CHAP	3-[(3-Cholamidopropyl)Dimethylammonio]-1-Propanesulfonate
FabG	3-Oxoacyl-[Acylcarrierprotein] Reductase
3PGA	3-Phosphoglycerate
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
ABE	Acetone-Butanol-Ethanol
ACN	Acetonitrile
Accc	Acetyl-Coa Carboxylase BC Subunit
FAB2, SSI2, DESA1	Acyl-[Acyl-Carrier-Protein] Desaturase
ATP	Adenosine Triphosphate
ATCC	American Type Culture Collection
ACL	ATP Citrate Lyase
BLAST	Basic Local Alignment Search Tool
BAM	Beta-Amylase
PB	Binding Buffer
BBM	Bold's Basal Medium
H_3BO_3	Boric Acid
BSA	Bovine Serum Albumin
$CaCl_2.2H_2O$	Calcium Chloride Dihydrate
CO_2	Carbon Dioxide
CCMs	Carbon Dioxide Concentrating Mechanisms
CO	Carbon Monoxide
CA	Carbonic Anhydrase
CCD	Central Composite Design
CCB	Centre For Chemical Biology
$Co(NO_3)_2.6H_2O$	Cobalt (Ii) Nitrate Hexahydrate
cDNA	Complementary Deoxyribonucleic Acid
$CuSO_4.5H_2O$	Copper (Ii) Sulfate Pentahydrate
DNA	Deoxyribonucleic Acid
DGAT	Diacylglycerol Acyltransferase

DIL	Dilution
K_2HPO_4	Dipotassium Hydrogen Phosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FDR	False Discovery Rate
FAD	Fatty Acid Desaturase
FAT	Fatty Acyl-Acp Thioesterase A
FPU	Filter Paper Units
FG	First Generation
DSMZ	German Collection Of Microorganisms And Cell Cultures
AGPase	Glucose-1-Phosphate Adenylyltransferase Small Subunit
G6PD	Glucose-6-Phosphate Dehydrogenase
3PGAL	Glyceraldehyde 3-Phosphate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
G/L	Gram Per Liter
HPLC	High Performance Liquid Chromatography
HI&I	Household, Industrial, And Institutional
H_2	Hydrogen
IAA	Indole-3-Acetic Acid
$FeSO_4.7H_2O$	Iron (Ii) Sulfate Heptahydrate
ISA	Isomaltase
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
ACSL	Long-Chain Acyl-Coa Synthetase
PL	Lysis Buffer
$MgSO_4.7H_2O$	Magnesium Sulphate
MCAT	Malonyl Coa-Acp Transacylase
$MnCl_2.4H_2O$	Manganese (Ii) Chloride Tetrahydrate
$MnSO_4.4H_2O$	Manganese Sulphate
m/z	Mass Over Charge Ratio
MJ/L	Megajoules Per Liter
mg/ L	Milligram Per Liter
MECR	Mitochondrial Trans-2-Enoyl-Coa Reductase
M-BPP	Modified Borax/ Polyvinvyl-Polypyrrolidone/ Phenol
M	Molarity

MoO_3	Molybdenum Trioxide
MSA	Multiple Sequence Alignment
ME	NADP-Dependent Malic Enzyme
NCBI	National Center For Biotechnology Information
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
N_2	Nitrogen
OPEFB	Oil Palm Empty Fruit Brunch
OD	Optical Density
O_2	Oxygen
PMSF	Phenylmethylsulfonylfluoride
PEPase	Phosphoenolpyruvate Carboxylase
PGM	Phosphoglucomutase
PBR	Photobioreactor
PS II	Photosystem II
KH_2PO_4	Potassium Dihydrogen Phosphate
KOH	Potassium Hydroxide
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RID	Refractive Index Detector
RC	Regenerated Cellulose
rpm	Revolutions Per Minute
RNA	Ribonucleic Acid
RuBP	Ribulose 1,5-Bisphosphate
RuBC	Ribulose Bisphosphate Carboxylase Oxygenase
SG	Second Generation
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
SCP	Single Cell Picking
Na_2CO_3	Sodium Carbonate
NaCl	Sodium Chloride
SDS	Sodium Dodecyl Sulfate
$NaNO_3$	Sodium Nitrate
SG	Starch Granules
SP	Starch Phosphorylase
SS	Starch Synthase

H_2SO_4	Sulfuric Acid
TG	Third Generation
TEM	Transmission Electron Microscope
TCA	Tricarboxylic Acid Cycle
TYA	Tryptone-Yeast-Extract-Acetate
$ZnSO_4.7H_2O$	Zinc Sulfate Heptahydrate

LIST OF APPENDICES

PEMERANGKAPAN CO_2 DAN BIOSINTESIS KARBOHIDRAT UNTUK PENGHASILAN BIOBUTANOL DARIPADA MICROALGA ASIDOFILIK PENCILAN TEMPATAN MELALUI PROSES SAKARIFIKASI DAN FERMENTASI SERENTAK

ABSTRAK

Butanol adalah bahan kimia yang biasa digunakan sebagai tambahan untuk bahan api automotif. Antara kaedah pengeluaran, biobutanol yang disintesis melalui proses penapaian aseton-butanol-etanol (ABE). Penapaian ABE menggunakan biojisim mikroalga yang mengandungi karbohidrat tinggi dan kurang lignin, adalah sesuai untuk dijadikan sebagai bahan mentah biobutanol. Potensi mikroalga untuk pemerangkapan CO_2 secara biologi, menjadikannya sebagai nilai tambah berbanding dengan sumber bahan mentah yang lain. Di samping itu, kombinasi kaedah sakarifikasi dan fermentasi (SSF) semasa proses penapaian ABE, telah membuka ruangan baharu dalam kemajuan pengeluaran biobutanol secara berekonomi. Berdasarkan kajian ini, sebanyak dua mikroalga asidofilik telah berjaya dipencilkan dan dikenal pasti sebagai strain *Coccomyxa dispar* dan *Scenedesmus parvus*. *C. dispar* dan *S. parvus* telah mempamerkan nilai tertinggi dalam produktiviti biojisim, produktiviti karbohidrat, dan pemerangkapan CO_2 apabila dibiak bawah keadaan CO_2 yang tinggi. Selain itu, gen dan protein yang berkaitan dengan biosintesis karbohidrat juga telah dikaji dalam kajian ini. Berdasarkan analisis transkriptomi, keputusan menunjukkan bahawa kenaikan yang signifikan bagi gen berkaitan dengan biosintesis karbohidrat seperti AGB, SS, ISA, AGPase, ME, G6PD, Accc, RuBC, dan CA yang terlibat dalam *C. dispar*, manakala PGM, AGB, SS, AGPase, ME, DGAT, RuBC, dan CA terlibat dalam *S. parvus*. Seterusnya, pengekstrakan protein

telah dilakukan bagi kedua-dua strain mikroalga dan mendapati kaedah boraks/polyvinvyl-polypyrrolidone/phenol (M-BPP) yang diubahsuai telah menghasilkan kadar protein yang tinggi iaitu 2.717 ± 0.032 mg/mL (2.520 ± 0.030 $mg/g_{biomass}$) dan 1.346 ± 0.011 mg/mL (1.299 ± 0.011 $mg/g_{biomass}$) daripada biojisim *C. dispar* dan *S. parvus*. Kebanyakan protein yang diekstrak bertanggungjawab untuk metabolisme tenaga, metabolisme karbohidrat, fotosintesis, dan proses selular (sitokeleton). Kemudian, kajian ini juga mendedahkan bahawa strain *C. dispar* dan *S. parvus* menunjukkan prestasi yang lebih baik di tanaman luaran dengan pengeluaran biojisim yang lebih tinggi sebanyak 20.43% dan 8.79% dengan produktiviti karbohidrat 52.80% dan 24.64% berbanding tanaman dalaman. Selain itu, kajian ini juga melaporkan bahawa kadar pemerangkapan CO_2 untuk *C. dispar* dan *S. parvus* adalah lebih tinggi berada di tanaman luaran berbanding dalaman. Akhir sekali, pengeluaran biobutanol yang optimum ialah 54.00 ± 3.25 dan 39.00 ± 2.28 $mg/g_{biojisim}$ dengan menggunakan biojisim daripada *C. dispar* dan *S. parvus* di bawah proses SSF. Hasil daripada kajian ini bermanfaat untuk mengurangkan pencemaran udara dan menghasilkan bahan kimia dengan menggunakan sumber bio boleh diperbaharui, yang boleh berguna untuk banyak aplikasi perindustrian.

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ABSTRACT

Butanol is a common chemical that used as an additive for automotive fuel. Among the production methods, the biobutanol synthesised through acetone-butanol-ethanol (ABE) fermentation process. The ABE fermentation using microalgae biomass that contains high carbohydrate with less lignin, which is suitable to be biobutanol feedstock. The potential of microalgae for biological CO_2 biosequestration, making them value-added compared to other bioresources. In addition, the promising single-step saccharification and fermentation (SSF) process during ABE fermentation, has opened up a novel ground for advancement in economic biobutanol production. Based on this study, a total of two native acidophilic microalgae were successfully isolated and were identified as *Coccomyxa dispar* and *Scenedesmus parvus* strains. The *C. dispar* and *S. parvus* exhibited highest in terms of biomass productivity, carbohydrate productivity, and CO_2 biofixation when cultivated under the elevated condition. Apart from that, the carbohydrate-related genes and proteins were also been investigated in this study. Based on the transcriptomic analysis, the results showed that a significant upregulated of carbohydrate-related genes such as AGB, SS, ISA, AGPase, ME, G6PD, Accc, RuBC, and CA that involved in *C. dispar*, while PGM, AGB, SS, AGPase, ME, DGAT, RuBC, and CA involved in *S. parvus*. Next, the protein

extraction was performed for both microalgae strains and it found out that the modified borax/ polyvinvyl-polypyrrolidone/ phenol (M-BPP) method could extract the highest protein yield of 2.717 ± 0.032 mg/mL (2.520 ± 0.030 $mg/g_{biomass}$) and 1.346 ± 0.011 mg/mL (1.299 ± 0.011 $mg/g_{biomass}$) from *C. dispar* and *S. parvus* biomass respectively. Most of the detected proteins were responsible for energy metabolism, carbohydrate metabolism, photosynthesis, and cellular processes (cytoskeleton). Later, this study also revealed that *C. dispar* and *S. parvus* strains exhibited a better performance under outdoor cultivation, with higher biomass production of 20.43% and 8.79% with carbohydrate productivity of 52.80% and 24.64% respectively compared to indoor cultivation. Apart from that, this study also reported that the CO_2 biofixation rate for *C. dispar* and *S. parvus* was higher under outdoor compared to indoor condition. Lastly, the optimum biobutanol production was 54.00 ± 3.25 and 39.00 ± 2.28 $mg/g_{biomass}$ using *C. dispar* and *S. parvus* biomass respectively under SSF process. The obtained value is higher compared to the previous studies. Hence, the outcome of this study was beneficial to reduce air pollution and producing fine chemicals using renewable bioresource, which could be useful for many industrial applications.

CHAPTER 1

INTRODUCTION

1.1 Research Background

The production of biofuels such as bioethanol, biogas, biohydrogen, and biobutanol from renewable feedstocks to meet the future energy demand, has gained the attention globally. Among of these biofuels mentioned, biobutanol has been identified as the next-generation transportation biofuel that has more advantages compared to bioethanol and biomethanol (Yeong et al., 2018a). The advantages of biobutanol include high octane number, high energy content, lower volatility, low vapor pressure, and flexible fuel blends. Apart from that, biobutanol is also has fewer ignition problems, possesses inter-solubility as well as higher viscosity and lubricity (Nanda et al., 2017; Trindade & Santos, 2017). Currently, butanol is produced through a chemical synthesis route as either oxo process from propylene (with H_2 and CO over a rhodium catalyst) or aldol process from acetaldehyde (Qureshi, 2009).

However, the traditional chemical synthesis of butanol has few major problems such as not cost-effective of unspecific catalyst using during the transformation process and resulting in low butanol yield. Apart from that, the application of high pressure and temperature up to 120-190 °C and 40-300 bar respectively during the large-scale butanol production also lead to the rising overall production cost (Zhang et al., 2022). Apart from that, the production of butanol via chemical reaction involved using petroleum-based feedstock which led to the continuous rising of the price. Unsatisfactory yield of butanol production has been the major driving factors for the increased interest in biological synthesis of biobutanol. Production of biobutanol via biological approach could reduce the price by using various renewable biomass such as sugarcane, sugar beet, sorghum, woody

crops, wheat straw, corn husks and microalgae biomass as feedstock to replace the overall chemicals usage (Kolesinska et al., 2019; Kushwaha et al., 2019; Lee & Lavoie, 2013; Ndaba et al., 2015).

Among the renewable biomass, microalgae biomass has been widely seen discussed and considered as one of the potential sources for biobutanol production through acetone-butanol-ethanol (ABE) fermentation by *Clostridium* sp. (Kolesinska et al., 2019; Onay, 1930; Yeong et al., 2018a). The utilization of microalgae biomass as biobutanol feedstock is more attractive than other typical types of renewable feedstock as it contains less lignin and have a simple cell structure (Khan et al., 2018a). Less lignin contain in microalgae biomass could contribute to the recovery effectiveness of pretreatment and fermentation processes prior the biobutanol production. In addition, high carbohydrate content in microalgae biomass has added its value by making as a promising feedstock for the biobutanol production. The presence of high carbohydrate content could minimise the industrially established operations such as extraction, purification, and concentrations steps for subsequently upgrading to higher-value biobutanol production (Wiatrowski et al., 2022). Previous study on biobutanol by *Clostridium acetobutylicum* using *Chlorella* sp. biomass treated with acidic hydrolysis (1mole of H_2SO_4) and autoclaving induced biobutanol up to 6.23 ± 0.19 g/L (Onay, 1930). Therefore, it was believed the obtained data could extent the current insight into the potential capability of using carbohydrate rich microalgae for biobutanol production, and this will be beneficial as a platform for the industrial scale application.

On the other hand, another advantage of these photosynthetic microalgae utilise sunlight and carbon dioxide (CO_2) as key regulators to perform photosynthesis

for their growth that could facilitate minimizing the carbon footprint (Onyeaka et al., 2021). These microalgae have an ability to develop an inducible CO_2 concentrating mechanisms (CCMs) in during photosynthetic metabolism and allow them to optimize CO_2 sequestration. The regulation of CCMs is also depended on the co-localization of carbonic anhydrase (CA) activity that located near the Rubisco to catalyse dehydration of bicarbonate from atmospheric CO_2, and provide near-saturating CO_2 concentrations for carboxylation of Ribulose 1,5-bisphosphate (RuBP) in Calvin cycle (Spalding, 2008). In addition, microalgae could exhibit higher nutrient uptake, which accumulate in cell vacuoles and promote a fast growth rate. This makes shorten the harvesting time compared to terrestrial plants that require more than 3 months before the biomass can be harvested (Dębowski et al., 2020; Harun & Danquah, 2011a; Paes et al., 2016a). Moreover, the biomass produced from cultivation contains valuable chemical compounds including carbohydrates, proteins, lipids, vitamins, pigments, and bioactive compounds (Khan et al., 2018a). All these chemical compounds can be subsequently be converted into high value-added products, like biofuel in which could be beneficial to many industry areas.

Dual CO_2 biofixation and biobutanol production are believed to be one of the potential approaches to achieve a sustainable biobutanol production. Cultivation of microalgae using CO_2 released from industrial activities to produce biomass for biobutanol fermentation could indirectly reduce the CO_2 concentration in atmosphere, resulting in reducing the greenhouse gas emissions. Unfortunately, this approach has caused the negative effect to the microalgae growth, as the presence of CO_2 in the cultivation medium could reduce the pH level. Then, the cultivation under the low pH condition could cause the alternation in carbohydrate polymer

biosynthesis mechanism at cellular level of certain species of microalgae. Cultivation using acidophilic microalgae could be an approach to overcome this limitation and ensure sustainable continuous biomass production.

To date, there are few studies have been conducted on the CO_2 biosequestration by different types of microalgae and its metabolites accumulation for biobutanol production. However, it is still remained unclear on carbohydrate biosynthesis mechanism in microalgae cultivated under CO_2 elevated condition. Also, there are limited information on the cultivation of extremophile microalgae strains in dual CO_2 biosequestration and biobutanol production. Therefore, this current study focuses on enhancing acidophilic microalgae on carbohydrate biosynthesis during cultivation process, and its potential as a feedstock for biobutanol production by *Clotridium* through ABE fermentation. The work is divided into three phases: the first phase focuses on the isolation of acidophilic microalgae from Bukit Katak and determination the best cultivation conditions such as pH, light intensity, temperature, and CO_2 concentration for maximal biomass production and carbohydrate accumulation. The investigation on the effect of CO_2 towards carbohydrate biosynthesis was also evaluated based on gene expression and proteomic analysis at microalgae cellular level. The second phase focuses on the upscaling of the acidophilic microalgae using 20L photobioreactor (PBR) for maximum microalgae biomass production. Lastly, the biomass produced was used for biobutanol production via novel simultaneous saccharification and fermentation (SSF) process under anaerobic fermentation.

1.2 Research Scope and Objectives

Overall, this research focuses the on isolation of novel acidophilic microalgae from Frog Hill, Pulau Pinang. The isolated strains were identified using molecular identification 18sRNA and phylogenetic tree. Subsequently, the strain was characterised for high biomass production and carbohydrate productivity based on different cultivation parameters. The genes, proteins, and enzymes that control the carbohydrate accumulation during the cultivation under CO_2 elevated condition of the isolated microalgae were characterised. This was followed by the large-scale cultivation of the selected microalgae under 20L photobioreactor was conducted to enhance the microalgae biomass and carbohydrate productivity. Lastly, the microalgae biomass was used as carbon source to synthesis the biobutanol through ABE fermentation by *Clostridium saccharoperbutylacetonicum* (N1-4).

The objectives of this study were:

I. To isolate the most tolerant with carbohydrate-rich acidophilic microalgae from Bukit Katak, Pulau Pinang.

II. To characterise the growth and carbohydrate productivity of acidophilic microalgae using different abiotic factors such as pH, light intensity, temperature and CO_2 concentrations (v/v).

III. To determine the correlation expression of key genes, enzymes, as well as level of metabolites implicated in carbohydrate biosynthesis pathways under elevated condition based on transcriptomic and proteomic analysis.

IV. To compare the biomass production and carbohydrate accumulation of the selected strains cultivated in large scale application (20L PBR) under indoor and outdoor conditions.

V. To optimise the pH, temperature, agitation speed, and solid loading on biobutanol production for *Clostridium saccharoperbutylacetonicum* (N1-4) using pretreated microalgae biomass as a carbon source.

CHAPTER 2

LITERATURE REVIEW

2.1 Classification of biofuel

Biofuels are a type of fuels that produced from renewable materials such as biomass, and potentially to be partially replaced the transportation fuel derived from petroleum-based feedstock. There are few examples of biofuels such as bioethanol, biobutanol, biodiesel, biohydrogen, and biomethane. Generally, biofuels can be produced from different feedstocks, which are first (FG), second (SG), and third generation (TG) of feedstock, depending on the types of biomass used (Figure 2.1).

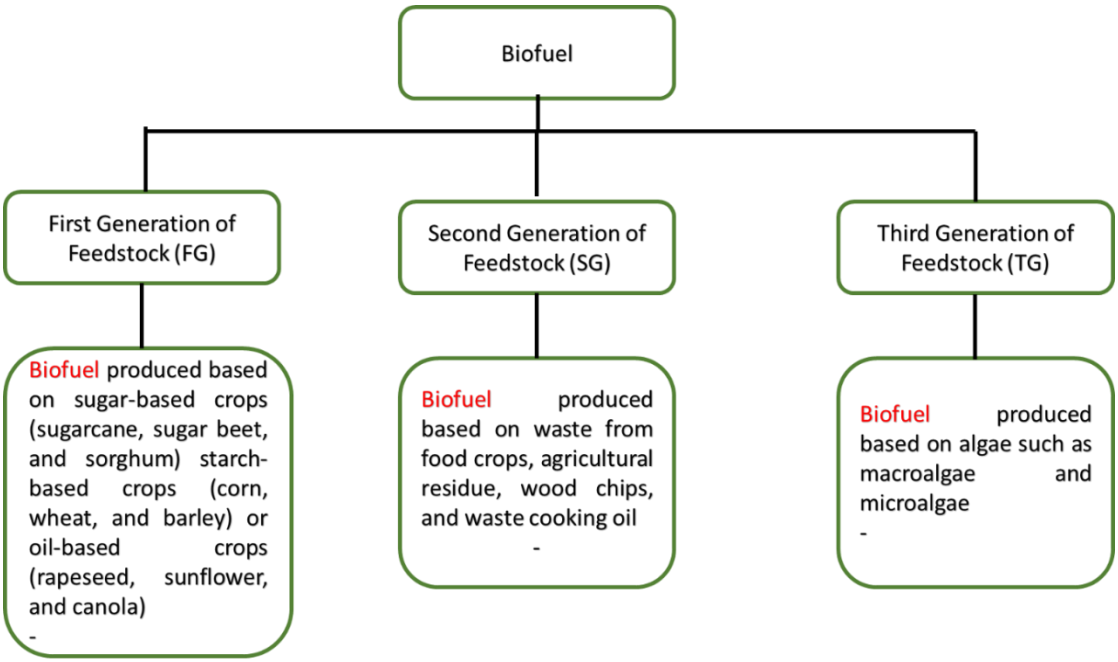

Figure 2.1 Classification different generations of biofuel feedstock (Sikarwar et al. 2017)

Generally, the FG are produced from starch, sugar, vegetable oil, or animal fats using conventional technology. However, the issues of generating the biofuel using first generation food crop were raised in last decade due to the social, environmental, economic and ethical challenges for using food crop as a carbon source (Mohr & Raman, 2013). Opposition to FG is commonly about the conflict with

food security. Then, the trend of producing biofuel was started using non-food crops, like cellulosic energy crop, waste biomass including wheat stalks, corn, and wood, which referred as SG (Lee & Lavoie, 2013). The SG are widely seen as a sustainable response to the increasing controversy surrounding FG, and thus using a non-food biomass as a carbon source ensures a sustainable alternative for biofuel production. Although the SG maybe more economically affordable than FG, but the technical feasibility due to thick lignin of SG is questioned. The issue such as few energy and sustainability have been raised about the SG in which this method would not economically favourable enough to stand as an alternative for non-renewable energy resources. Previous study showed that the biofuel production using the SG biomass such as waste from food crop, agriculture residue, and wood chip that consisted of thick lignin content (25-35% dry weight) have impeded the downstream processing for biofuel production especially in the pretreatment process to unlock the sugar embedded in the biomass (Lee & Lavoie, 2013). Apparently, the sophisticated process such as pretreatment is compulsory applied during downstream process prior the biofuel production will cause the overall cost not effective for the commercial production.

TG on the other hand is believed could help to overcome the above situation. Generally, the TG are produced from photosynthetic micro-organisms such as algae. The potential of microalgae which could produce high biomass productivity, and able to cultivated on non-arable land, with not competing with food production system (Li-Beisson & Peltier, 2013). Another notable advantage of microalgae is this microorganism could reduce the carbon dioxide (CO_2) emission from the atmosphere and converted into usable macromolecule such as carbohydrates or lipids through the photosynthesis metabolism process (Li-Beisson & Peltier, 2013). Currently, TG are

still under development and few studies have showed the potential of using microalgae biomass for biofuel production (Nagi et al., 2020; Walmsley et al., 2018; Zewdie & Ali, 2020). Therefore, further researches are needed to further minimize the microalgae cultivation time, nutrient cost and extraction process in order to make it economically competitive to petrodiesel, petroleum-based fuels, and fine chemicals production.

To date, there are few types of biofuels are available such as bioethanol, biobutanol, biodiesel, and bio-oil which derived from TG. Among the biofuel, the biobutanol showed the privilege than others, as an advanced for next-generation transportation biofuel. This was due to the characteristics of butanol have high energy content as gasoline and can used directly without modifying current internal combustion engines (Xue & Cheng, 2019b). At this stage, the production of butanol still below the satisfactory level, to sustain the estimated global market size demand around 6.17 million metric tons during year 2026 (Baron, 2022). Currently, the butanol production is still expensive due to the high cost of feedstock, process application, and undesirable selectivity. Hence, the considerable effort should be imposed to overcome this current issue.

2.2 Butanol as next generation biofuel

Butanol also known as butyl alcohol or n-butanol, is a four-carbon alcohol with a chemical formula of C_4H_9OH. Generally, butanol is a colourless liquid and miscible in organic solvent, while partially miscible in water. The characteristic of butanol is shown in Table 2.1.

Table 2.1 Properties of butanol (Gowtham et al., 2019)

Properties	Butanol
Chemical formula	C_4H_9OH
Molecular weight (g/mol)	74.12
Cetane number	15.92
Low heating value (MJ/kg)	42.16
Heat of evaporation (kJ/kg)	312
Stoichiometric air–fuel ratio	11.21
Auto-ignition temperature	421
Research Octane number	94
Calorific Value MJ/kg	42
Flash point °C	38
Density kg/m3 @20 °C	762

This chemical is widely used in various industries especially in chemical industries. Butanol can be used as an organic solvent and also as an intermediate component in the manufacture of other organic chemicals such as butyl acetate and butyl glycol ethers. Both organic chemicals are specialty solvents in household, industrial, and institutional (HI&I) cleaning applications (Baker, 2015). Apart from that, butanol is also commonly used in pharmaceutical industry for manufacturing of antibiotics, hormones, and vitamins.

Butanol is considered superior to other biofuels such as ethanol as it comprises two times number of carbon atoms compared to ethanol, leading to its higher higher energy density (Ndaba et al., 2015). It can also be used as fuel addictive that can be blended directly with gasoline and used in the internal combustion engine without any modification (Bhatia, 2014). The high energy density of 29.2 MJ/L (compared to 19.5 MJ/L of ethanol and 16MJ/L of methanol), and can replace gasoline (energy density 32MJ/L). The butanol has less hygroscopic characteristic and less corrosive compared to ethanol, which is safer and transported easily in pipelines (Xue & Cheng, 2019a). The low volatility of butanol also makes it less explosive than ethanol (Liu et al., 2013). A comparison of fuel characteristics is shown in Table 2.2**.**

Table 2.2 Comparison characteristics between gasoline, butanol, and ethanol (Ndaba et al., 2015; Zhuang et al., 2013)

Characteristics	Gasoline	Butanol	Ethanol
Air-fuel ratio	14.70	11.10	9.00
Energy density (MJ/ kg)	32.00	29.20	19.50
Specific energy (MJ/kg air)	2.90	3.20	3.00
Heat vaporisation (MJ/kg)	0.36	0.43	0.92

2.2.1 Butanol production technology

Butanol can be produced through the chemical and biological routes. In chemical synthesis, butanol is synthesised based on oxo synthesis, Reppe synthesis or crotonaldehyde hydrogenation. In biological conversion, it is produced through acetone-butanol-ethanol (ABE) fermentation using bioresource rich carbohydrate as a feedstock. In this process, the carbohydrate will be converted into monomer (sugar) by enzymatic hydrolysis prior fermented into biobutanol with the aid of bacteria *Clostridium* sp. under anaerobic condition (Guo et al., 2022).

2.2.1(a) Chemical synthesis of butanol

Generally, the oxo synthesis involves two steps of reactions. In the first step of reaction, the propylene ($CH_3CH=CH_2$) is reacted with carbon monoxide (CO) and hydrogen (H_2) in the presence of catalyst such as cobalt or rhodium. Mixture of n- and isobutyraldehyde is then hydrogenated to n- and isobutyl alcohols. This is followed by then further proceed with distillation step to recover butanol as shown in the Equation 2.1 and 2.2 (Ravichandra et al., 2019).

$$\underset{\text{Propylene}}{CH_3CH=CH_2} \xrightarrow[CO+H_2]{yields} CH_3CH_2CH_2CHO + (CH_3)_2CHCHO \qquad \text{Eq. 2.1}$$

$$CH_3CH_2CH_2CHO \xrightarrow[H_2]{yields} \underset{\text{Butanol}}{CH_3CH_2CH_2CH_2OH} \qquad \text{Eq. 2.2}$$

In Reppe process or also known as carbonylation process, butanol is produced directly under low pressure and temperature condition. In this reaction, propylene is reacted with carbon monoxide and water under low pressure 0.5-2 x 106 Pa and of 100 °C in the presence of a catalyst. The common catalyst used in this process is iron carbonyls (Sons, 2000) as shown in the Equation 2.3. However, Reppe process was found not as famous as oxo synthesis due to the high cost processing technology is required and have limited in commercialization (Jain et al., 2014).

$$\underset{\text{Propylene}}{CH_3CH{=}CH_2} \xrightarrow[CO+H_2]{yields} \underset{\text{Butanol}}{CH_3CH_2CH_2CH_2OH} + (CH_3)_2CHCH_2OH + 2CO_2 \qquad \text{Eq. 2.3}$$

In crotonaldehyde hydrogenation process, the reactions involved are aldol condensation, dehydration and hydrogenation. Different from other chemical synthesis that solely depend on petroleum, the crotonaldehyde hydrogenation process provides an alternative route for the butanol production from ethanol, which can be ubiquitously produced from biomass. In this process, the reaction is started with aldol condensation process of acetaldehyde (CH_3CHO), followed by hydrogenation to produce butanol. The dehydration process also been taken place in which induced by acidification, using an acid such as acetic acid or phosphoric acid Generally, this process is performed in a liquid phase under ambient temperature and pressure in the presence of an alkaline catalyst as shown in the equation 2.4 and 2.5.

$$\underset{\text{Acetaldehyde}}{2CH_3CHO_2} + H_2 \xrightarrow[\text{Aldol condensation}]{yields} CH_3CH(OH)CH_2CHO \xrightarrow[\text{Dehydration}]{yields} CH_3CH{=}CHCHO + H_2O \qquad \text{Eq. 2.4}$$

$$CH_3CH{=}CHCHO + H_2 \xrightarrow[\text{Hydrogenation}]{yields} \underset{\text{Butanol}}{CH_3CH_2CH_2OH} \qquad \text{Eq. 2.5}$$

However, due to the economic and sustainability reasons, the butanol produced from the above methods cannot applied as alternative fuel components (Kolesinska et

al., 2019). Hence, this led to the establishment of other alternative butanol approaches such as acetone-butanol-ethanol (ABE) fermentation to facilitate butanol production under cheaper and sustainable manner.

2.2.1(b) Biological approach for acetone-butanol-ethanol (ABE) fermentation of butanol

Another approach to produce butanol is through acetone-butanol-ethanol (ABE) fermentation. This process uses bacterial fermentation to produce solvents such as acetone, butanol, and ethanol from carbohydrates polymers such as starch and glucose under anaerobic condition. During this process, the bacteria will convert the fermentable sugar/glucose into the ABE products in a mass ratio of 3:6:1 which follow the Equation 2.6 below:

$$\underset{\text{Starch}}{(C_6H_{10}O_5)_{10}} + 9H_2O \xrightarrow[+\ Biomass]{yields} \underset{\text{Acetone}}{3C_3H_6O} + \underset{\text{Butanol}}{6C_4H_{10}O} + \underset{\text{Ethanol}}{C_2H_6O} + 24CO_2 + 16H_2 \quad \text{Eq. 2.6}$$

One of the most common bacteria used in the ABE fermentation is *Clostridium* sp. Generally, *Clostridium* is gram-positive, rod shaped, with cell size diameter varies from 0.3 to 2.0 um, while the lengths from 1.5 to 2.0 um (Liberato et al., 2019). The bacteria cells are normally arranged in pairs or in short chains with rounded or pointed ends. Most of the *Clostridium* species have sporulation capacity, which can be triggered and turned into spore in its growth cycle by presence of oxygen. However, the species of *Clostridium* species have different oxygen tolerance levels, has a capability to degrade a wide range of polysaccharides and converted it into solvents as well as organic acid, making it potential to be used for biobutanol production (Liberato et al., 2019).

Typically, ABE fermentation occurred in *Clostridium* sp. involves two different stages, (i) acidogenesis that responsible for sugar conversion into organic

acid, and (ii) solventogenesis that responsible for solvent production (Kolesinska et al., 2019) (Figure 2.2). During the acidogenesis phase, the bacteria grow drastically and producing acids (mostly acetate and butyrate). The accumulation of acid products in the medium will lead to a decrease in pH to around 4.5. However, towards the end of acidogenesis, the acid production rate falls due to the bacterial cells shift their metabolic activity from acidogenesis to solventogenesis (Daniel et al., 2011). In this phase, both acetate and butyrate are being consumed as substrates for the biosynthesis of acetone and butanol, and the bacteria will undergo stationary stage under this phase. At the end of solventogenesis phase, the concentrations of butanol and other products reaches a level which ceases all bacterial metabolism (Bowles & Ellefson, 1985).

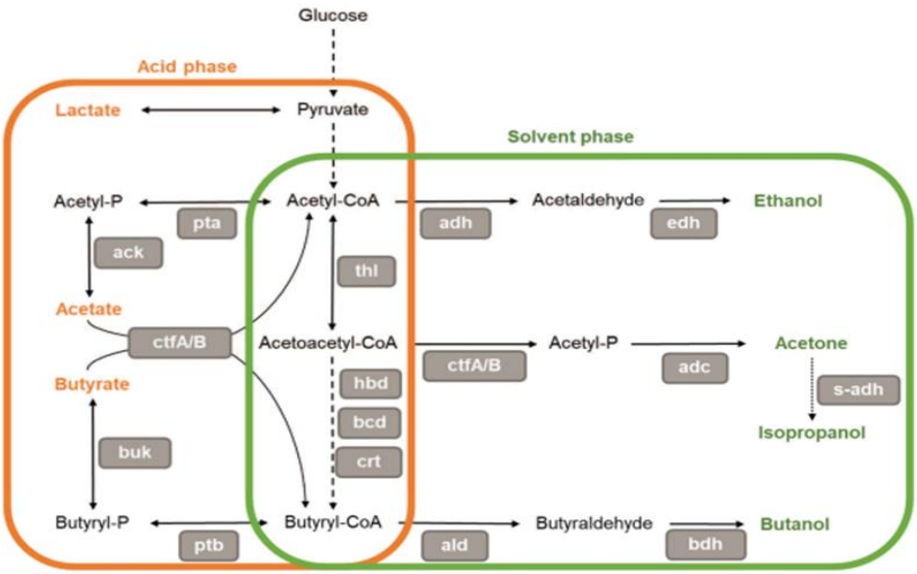

Figure 2.2 Simplified acetone-butanol-ethanol metabolic pathway in solventogenic *Clostridia* sp. (Diallo et al., 2021)

2.3 ABE fermentation feedstocks

Feedstock selection is an important factor to ensure the economic feasibility for butanol production via fermentation process, where the substrate cost is typically equating to more than 60% of the overall biobutanol production cost (Dürre &

Eikmanns, 2015). By selecting a low-cost substrate such as renewable bioresources are essential for this fermentation to occur. Several studies have been conducted to identify the suitable feedstock to produce biobutanol (Table 2.3). Based on the Table 2.3, it was found that different substrates could give different percentages of butanol production. For instance, Jerusalem artichoke, sago starch, wheat straw, barley straw, cassava chip able to produce a desirable amount of butanol concentration as 12.00 to 18.00 g/L. On the other hand, the lowest butanol concentration 2.80 g/L was produced using the oil palm empty fruit bunch as a substrate. From this analysis, the butanol production can be affected by the selection of substrate materials. The complexity of the cell wall, membrane, and chemical compositions of the substrates could affect the overall pretreatment process and hence affecting the butanol production.

Table 2.3 Biobutanol production using different substrates

Substrates	Bacteria	Butanol concentration (g/L)	References
Soy molasses	*Clostridium beijerinckii* BA 101	8.00	(Li et al., 2020)
Jerusalem artichoke	*Clostridium acetobutylicum* IFP 904	14.80	(Li et al., 2020)
Sago starch	*Clostridium acetobutylicum* P 262	16.00	(Li et al., 2020)
Domestic organic waste	*Clostridium acetobutylicum* ATCC 284	3.00	(Li et al., 2020)
Wheat straw	*Clostridium beijerinckii* P 260	12.00	(Li et al., 2020)
Barley straw	*Clostridium beijerinckii* P 260	18.00	(Li et al., 2020)
Glycerol and glucose	*Clostridium acetobutylicum* ATCC 4259	8.60	(Li et al., 2020)
Corn stover	*Clostridium beijerinckii* P260	8.98	(Qureshi et al., 2014)
Oil palm empty fruit bunch	*Clostridium acetobutylicum* ATCC 824	2.80	(Ibrahim et al., 2015)
Cassava chip	*Clostridium saccharoperbutylacetonicum* N1–4	16.40	(Thang et al., 2010)

Even though most of the studies showed the potential of aforementioned substrates for butanol production, however the problems of this feedstock have been identified in which high in overall cost due to complexity of the lignocellulosic material and high energy are required to break down the complexity during pretreatment process. All these drawbacks have led to the new approach to explore a new type of alternative substrate. Currently, the biobutanol production from renewable biomass such as microalgae has been explored as butanol feedstock.

2.4 ABE fermentation using microalgae biomass

Microalgae biomass have been identified could be used as biobutanol feedstock. The utilisation of microalgae biomass that is not competing with the food applications, containing less lignin which are found could overcome the current limitations. The prime advantage for the microalgae biomass is this feedstock containing high carbon in carbohydrate polymer is beneficial for ABE fermentation process (Linacre et al., 2021). Apart from that, microalgae which are microscopic photosynthetic microorganisms can use sunlight and available carbon dioxide (CO_2) to conduct photosynthesis for their growth. This would make the microalgae to exhibit higher nutrient uptake, and accumulated the obtained nutrients in the cell vacuoles. Subsequently, the microalgae have a fast growth rate. Normally, the microalgae harvesting time is less than 15 days compared to terrestrial plants that require more than three months before the biomass can be harvested (Harun & Danquah, 2011b; Paes et al., 2016b). In addition, the most important criteria of selecting microalgae biomass as biobutanol feedstock is that the microalgae biomass produced during cultivation period contains valuable chemical compounds including carbohydrates, proteins, and lipids. Previous studies show the microalgae biomass contains 5.4–

73.1% proteins, 1.5-52.0% carbohydrates, and 0.3-39.6% (dry weight basis) lipids depending on the microalgae species (Table 2.4). For the microalgae that possess high protein content can be converted into animal feed, cosmetic products, bio-fertiliser, and bioactive compounds, especially in the pharmaceutical industry (Milledge, 2011). Whereas, for the microalgae consists of high lipid content can be converted into biofuel, biodiesel, and bio-char. Lastly, the extracted microalgae carbohydrate was potential to be converted into bioethanol, bio-plastic, and fine chemicals production (Tan et al., 2021). Therefore, it can be concluded that the potential of high value-added products synthesised from microalgal biomass is totally dependent on the chemical composition within microalgae.

Table 2.4 Chemical composition of different microalgae species on a dry matter basis (%)

Microalgae species	Protein	Carbohydrate	Lipid	References
Anabaena cylindrical	43–56	25–30	4–7	(Um & Kim, 2009)
Aphanizomenon flosaquae	62	23	3	(Jayashree R et al., 2013)
Bellerochea sp.	14.2	3.01	9.87	(Costard et al., 2012)
Caulerpa lentillifera	10.41	38.66	1.11	(Tibbetts et al., 2015)
Chaetoceros sp.	10.5	1.50	3.73	(Costard et al., 2012)
Chlamydomonas rheinhardii	48	17	21	(Jayashree R et al., 2013)
Chlorella protothecoides (CS-41)	25.6	10.8	12.8	(Kumar et al., 2017)
Chlorella pyrenoidosa	57	26	2	(Um & Kim, 2009)
Chlorella sp.	6.07	7.09	1.82	(Costard et al., 2012)
Chlorella sp. (CS-247)	15.4	11	18.4	(Kumar et al., 2017)
Chlorella sp. (CS-195)	19	5.4	17	(Kumar et al., 2017)
Chlorella vulgaris	34.56	41.09	28.20	(Kumar et al., 2017)
Dunaliella salina	57	31.6	6.4	(Srinivasan et al.,

				2018)
Euglena gracilis	39–61	14-18	14-20	(Um & Kim, 2009)
Micromonas pusilla (temperate) (CS-98)	17.7	13.3	13.3	(Kumar et al., 2017)
Micromonas pusilla (tropical) (CS-170)	5.5	16.7	10.9	(Kumar et al., 2017)
Pyramimonas cordata (CS-140)	16.3	17	9.5	(Kumar et al., 2017)
Pycnococcus provasolii (CS-185)	17.3	13.8	16.7	(Kumar et al., 2017)
Rhodomonas sp.	44.9	8.60	39.2	(Costard et al., 2012)
Scenedesmus dimorphus	8–18	21–52	16–40	(Um & Kim, 2009)
Scenedesmus obliquus	50–56	10–17	12–14	(Um & Kim, 2009)
Stichococcus sp. (CS-92)	22.5	16.1	8.5	(Kumar et al., 2017)
Tetraselmis maculate	52	15	2.9	(Srinivasan et al., 2018)
Thalassiosira sp.	73.1	51.4	39.6	(Costard et al., 2012)
Tetraselmis chui (CS-26)	18.1	13.9	13.9	(Kumar et al., 2017)

2.5 Microalgae biomass pretreatment

Microalgae are eukaryotic microorganisms that consists of a complex polymer cell wall structure that mainly made up from non-cellulosic polysaccharides, such as galactose, rhamnose, glucuronic acid, and glucosamine, whereas glucose is only a minor component (Yee-Keung & Kin-Chung, 2020). These components play an important role in the formation of rigid microalgae cell structure that allows microalgae to maintain cell bodies under harsh conditions. Apart from maintaining cell structure, the polysaccharides are also important energy storage in microalgae cell (Moreira et al., 2022). On the other hand, the polysaccharide can also be extracted for various industrial applications such as food, cosmetic, pharmaceutical, and fine chemicals industries (Udayan et al., 2017).

Among of the valuable products that extracted from microalgae polysaccharides, Yeong et al. (2018b) showed that the biobutanol from fine chemical industry, was potential biosynthesised using microalgae in industrial scale through advances in bioprocess technologies. Hence, in order to extract the carbohydrates or polysaccharides from intracellular microalgal cells for biobutanol production, pretreatment step is required for breaking down this rigid microalgal cell wall structure, prior to series of bioprocess technologies such as enzymatic saccharification and fermentation process (Figure 2.3).

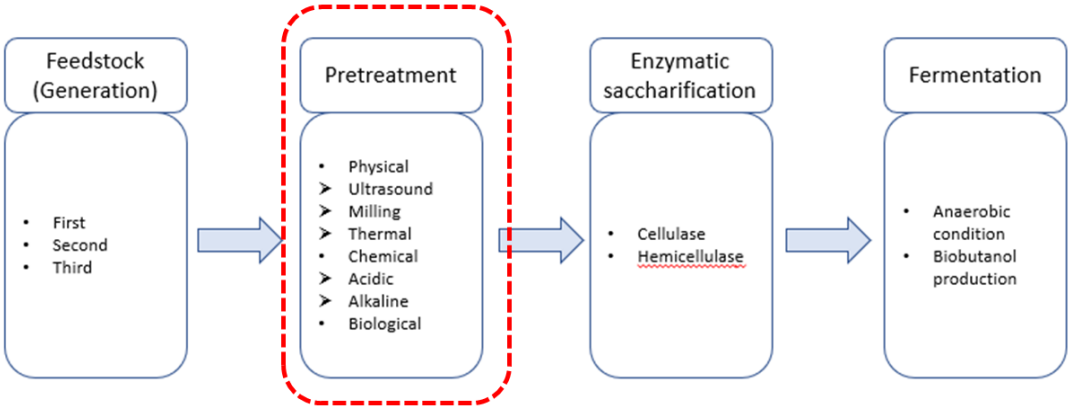

Figure 2.3 Flow diagram for biobutanol production from renewable feedstocks

The main aim of pretreatment is to enhance the accessibility for enzymatic hydrolysis and improves the carbohydrates or polysaccharides digestibility that are available in the biomass (Kucharska et al., 2018). To date, several microalgal cell pretreatment methods have been proposed by previous studies, and can be categorised into three main groups: physical, chemical, and biological (El-Dalatony et al., 2017). Generally, the physical pretreatment is used to reduce the particle sizes of the microalgal biomass, and increase the specific surface area by reducing the cellulose crystallinity (El-Dalatony et al., 2017). Whereas, the chemical pretreatment is a process that involved chemicals, either acids or alkaline to degrade the ester and glycosidic side chains bonding. This will result in lignin structural alteration, cellulose

swelling, partial decrystallization, and hemicellulose solvation (Brodeur et al., 2011). Lastly, the biological pretreatment is emphasised on the utilisation of microbes and enzymes to break down the biomass and release the simple sugars for the subsequent fermentation (El-Dalatony et al., 2017). The detail description of advantages and limitations for different pretreatment methods were summarised in Table 2.5.

Table 2.5 Types of pretreatment, advantages and limitations used to pretreat the microalgal biomass.

Types of Pretreatments	Advantages	Limitations	References
Physical			
➢ Ultrasound	➢ Ultrasonic wave creates a series of microbubble cavitation disrupt the microalgae cell structure ➢ Environmentally friendly method ➢ Require a short period of time ➢ Require low temperature ➢ Less chemicals usage	➢ High energy consumption ➢ Non-specific reaction ➢ Applicable for small biomass volume, not feasible for industrial scale ➢ Expensive cost for large scale	(Silva et al., 2018; Kim et al., 2014a)
➢ Bead beating (milling)	➢ Milling disrupt the cell membrane through grinding ➢ Could increase the surface area of the biomass ➢ Could reduce the crystallinity of cellulose for better hydrolysis	➢ High energy consumption ➢ Time-consuming process	(Alavijeh et al., 2020; Kim et al., 2014a)
➢ Thermal	➢ The heat introduced into the system ➢ Solubilise the cell wall of biomass ➢ Disrupt the whole microalgae structure ➢ Could increase the biomass load	➢ High energy consumption ➢ Less effective for microalgae with a simple cell wall structure	(Mendez et al., 2014; Rincón-Pérez et al., 2020)
Chemical			
➢ Acidic	➢ Concentrated acid disrupts the hydrogen bonds in the microalgal cell wall structure ➢ Provide higher efficiency in converting	➢ Involved the uses of chemicals ➢ Non-environmentally friendly	(Laurens et al., 2015)

	cellulosic materials.	➢ Formation of inhibitors ➢ High costs of corrosive resistant equipment ➢ High costs for recovery process	
➢ Alkaline	➢ Breaking the ester bond in the microalgal cell wall structure ➢ Effective on biomasses with low lignin ➢ Enlarges the surface area of cellulose by biomass swelling ➢ Reduced cellulose crystallinity by cleavage of carbohydrates glycosidic bond ➢ Less inhibitors that hampered the end product formation ➢ Environmentally friendly by using low concentration of alkali	➢ High costs alkaline catalyst ➢ Alteration of lignin structure	(Kassim & Bhattacharya, 2016; Mahdy et al., 2014b; OK et al., 2013)
➢ Biological	➢ Utilisation of microbes and enzymes that act as biocatalysts to degrade the microalgal cell wall ➢ Involved less toxic chemicals	➢ Required longer time ➢ Required high enzyme-to-substrate specificity	(Eldalatony et al., 2016; Fu et al., 2010; Kassim & Bhattacharya, 2016; Laurens et al., 2015;

	➢ Not energy intensive ➢ Involved specific reaction ➢ Do not required expensive equipment ➢ Easier for selective product recovery process	➢ Involved costly enzyme	Mahdy et al., 2014a)

2.6 Enzymatic saccharification

In order to recover the sugar from microalgae carbohydrate polymer, the pretreated biomass required to undergo enzymatic saccharification process. In this process, the microalgae carbohydrates contained in microalgal biomass are broken down into monomer sugars prior fermentation process. The utilisation enzymes for saccharification significantly depends on the microalgae cell wall composition, microalgae structure, and biochemical distribution. All these factors vary from each of the microalgae species (Tan et al., 2021).

The most common enzymes used in saccharification process are amylase and cellulase (Vasić et al., 2021; Vingiani et al., 2019). Several studies on enzymatic saccharification on different microalgae biomass such as *Scenedesmus* sp., *Chlorella* sp., *Nannochloropsis gaditana*, and *Tetraselmis suecica*, has been reported (Mahdy et al., 2016; Saleh et al., 2019; Ngamsirisomsakul et al., 2019). Amylase and cellulase are widely used to saccharify the intracellular cellulose and hemicellulose from cell wall structure in microalgal biomass and produce simple sugars for fermentation.

Theoretically, the efficiency of enzymatic saccharification of microalgal biomass for maximum sugar production prior to fermentation process are significantly depending on various saccharification parameters, including pH, temperature, enzyme loading, and biomass concentration (Choi et al., 2010; Vasić et al., 2021). Previous study found that the optimal enzymatic saccharification conditions of *Chlamydomonas reinhardtii* were achieved by employed 0.2% glucoamylase at temperature of 55°C and initial pH of 4.5. Another study also showed that the maximum sugars obtained up to 64% from *Chlamydomonas humicola* when applied the optimum saccharification process of 10 g/ L biomass at 40°C and an initial pH of 4.8 (Agbor et al., 2011).