



Faculty of Resource Science and Technology

**Total Sago Effluent Concentrate (TSEC) for Bioethanol Production via
Fed-Batch Simultaneous Saccharification and Fermentation (SSF)**

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Total Sago Effluent Concentrate (TSEC) for Bioethanol Production via Fed-Batch Simultaneous Saccharification and Fermentation (SSF)

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.


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ABSTRACT

The extraction of sago in sago processing industries requires substantial volumes of water, which subsequently generates large quantities of sago residue. This waste is often discharged into nearby streams, contributing to environmental pollution. Sago processing waste stream, or sago effluent, comprises a mixture of wet solid biomass and liquid hydrolysate. This industrial waste is rich in carbohydrates, particularly lignocellulosic materials, making it a potential feedstock for bioethanol production. In this study, the sago effluent was pretreated physically using mechanical pulverisation and centrifugation to produce a starch-rich wet biomass referred to as Total Sago Effluent Concentrate (TSEC). The TSEC was then utilised as a feedstock for bioethanol production in addition to enhance the utilisation of lignocellulosic materials from sago residue as well as to minimise unethical disposal of valuable resources. Compositional analyses were conducted on the TSEC prior to the enzymatic saccharification and fermentation process. Starch and fibre analyses were conducted using the Phenol-sulfuric Acid (PSA) assay, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF), Klason Lignin determination and standard protocol for ash content. Based on the results, TSEC comprised of $88.11 \pm 2.09\%$ sago effluent hydrolysate and $3.56 \pm 0.80\%$ sago hampas. The TSEC was subjected to further compositional analyses whereby $1.31 \pm 0.05\%$ of free starch was found in the sago effluent hydrolysate whilst, solid component was consisted of $65.87 \pm 4.25\%$ starch, $5.95 \pm 1.53\%$ cellulose, $18.20 \pm 1.60\%$ hemicellulose, $5.25 \pm 0.70\%$ lignin and $0.58 \pm 0.40\%$ ash. These compositional values suggest that the TSEC is a desirable feedstock for bioethanol production via fed-batch simultaneous saccharification and fermentation (SSF) catalysed by amylolytic enzymes (α -amylase and glucoamylase) and fermenting yeast, *Saccharomyces cerevisiae*. The SSF studies were conducted via fed-batch fermentation in

both non-buffered and buffered broth with varying strengths (25 mM, 50 mM and 100 mM) of citrate buffer at different biomass concentrations. Results indicated that SSF on TSEC yielded maximum ethanol concentration of 23.44 ± 4.16 g/L with the Theoretical Ethanol Yield (TEY) of $62.94 \pm 11.18\%$ in the non-buffered broth. There was no significant difference in ethanol produced in the different buffered broths. The highest ethanol concentration detected in 25 mM was 23.22 ± 2.44 g/L or $62.33 \pm 6.57\%$ TEY, followed by $55.33 \pm 4.80\%$ TEY (20.67 ± 2.85 g/L) in 50 mM. The highest ethanol yield detected in the sample buffered by 100 mM citrate buffer was at 20.61 ± 1.79 g/L with equivalent TEY of $55.33 \pm 4.82\%$. This study concludes that TSEC is viable feedstock for bioethanol production via the fed-batch SSF process in a non-buffered fermentation system.

Keywords: Bioethanol; simultaneous saccharification and fermentation; total sago effluent concentrate; fed-batch fermentation; *Saccharomyces cerevisiae*

***Keseluruhan Efluen Sagu Pekat (TSEC) untuk Penghasilan Bioetanol melalui
Sakrafikasi dan Fermentasi Serentak Berkala (SSF) secara Berkelompok***

ABSTRAK

Dalam industri kanji sagu, air yang banyak amat diperlukan dalam proses pengekstrakan. Prosedur ini menghasilkan banyak sisa sagu yang biasanya dibuang ke aliran berdekatan sehingga mengakibatkan pencemaran alam sekitar. Sisa sagu, atau efluen sagu, adalah campuran komponen pepejal basah dan komponen cair yang mengandungi karbohidrat dalam bentuk bahan lignoselulosa iaitu sumber yang bermanfaat dalam penghasilan bioetanol. Oleh itu, dalam kajian ini, sisa sagu diprarawat melalui proses pengisaran konstituen dan sentrifugasi untuk mendapatkan komponen pepejal berkanji tinggi yang disebut sebagai Keseluruhan Efluen Sagu Pekat (TSEC). TSEC digunakan sebagai substrat dalam produksi bioetanol di samping meningkatkan penggunaan bahan lignoselulosa daripada sisa sagu dan juga untuk meminimumkan pembuangan sumber bernilai secara tidak beretika. Analisa komposisi dilakukan pada TSEC sebelum proses sakrifikasi dan fermentasi enzimatik. Analisa kanji dan serat dilakukan menggunakan ujian Asid Fenol Sulfurik (PSA), Serat Detergen Asid (ADF), Serat Detergen Neutral (NDF), kajian Klason Lignin dan protokol standard untuk kandungan abu. Berdasarkan analisis, TSEC terdiri daripada $88.11 \pm 2.09\%$ hidrolisat efluen sagu dan $3.56 \pm 0.80\%$ hampas sagu. TSEC menjalani analisis komposisi lebih lanjut di mana $1.31 \pm 0.05\%$ kanji dijumpai dalam hidrolisat efluen sagu sementara komponen pepejal hampas sagu terdiri dari $65.87 \pm 4.25\%$ kanji, $5.95 \pm 1.53\%$ selulosa, $18.20 \pm 1.60\%$ hemiselulosa, $5.25 \pm 0.70\%$ lignin dan $0.58 \pm 0.40\%$ abu. Ini menunjukkan bahawa dalam kajian ini, TSEC adalah bahan mentah yang berpotensi untuk penghasilan bioetanol melalui sakrifikasi dan fermentasi serentak (SSF) berkala secara berkelompok dengan bantuan enzim amilolitik (α -amilase dan

glukoamilase) dan *yeast* fermentasi, Saccharomyces cerevisiae. SSF dilakukan melalui fermentasi serentak berkala secara berkelompok menggunakan sistem tanpa penampungan dan penampungan dengan menggunakan kekuatan penampungan sitrat yang berbeda (25 mM, 50 mM dan 100 mM) di dalam kepekatan biojisim yang berbeda. Hasil kajian menunjukkan bahawa penggunaan TSEC sebagai bahan mentah fermentasi menghasilkan bioetanol maksimum 23.44 ± 4.16 g/L dengan Hasil Etanol Teori (TEY) sebanyak $62.94 \pm 11.18\%$ dalam sistem tanpa penampungan. Tidak ada perbezaan yang signifikan dibandingkan dengan sistem yang mempunyai penampungan di mana yang tertinggi dalam 25 mM adalah 23.22 ± 2.44 g/L atau $62.33 \pm 6.57\%$ TEY dan diikuti oleh $55.33 \pm 4.80\%$ TEY (20.67 ± 2.85 g/L) dalam 50 mM. Yang terakhir, pengeluaran hasil etanol tertinggi yang dikesan dalam 100 mM adalah pada 20.61 ± 1.79 g/L dengan TEY setara $55.33 \pm 4.82\%$. Kesimpulannya, kajian kami menunjukkan TSEC adalah bahan mentah yang sangat berpotensi untuk penghasilan bioetanol melalui sakarifikasi dan fermentasi serentak berkala secara berkelompok.

Kata kunci: Bioetanol, sakarifikasi dan fermentasi serentak berkala (SSF), keseluruhan efluen sagu pekat (TSEC), fermentasi secara berkelompok, Saccharomyces cerevisiae

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significant differences via Tukey's *post hoc* test, $\alpha=0.05$]

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BOD	Biological Oxygen Demand
$C_6H_{12}O_6$	Glucose
C_2H_5OH	Ethanol
CO_2	Carbon
COD	Chemical Oxygen Demand
EU	European Union
g	gram
g/L	gram per liter
GMO	Genetically Modified Organisms
GRAS	Generally Recognized as Safe
h	hour
HPLC	High Performance Liquid Chromatography
IEA	International Energy Agency
m/L	mililiter
mM	milimolar
nm	nanometer
rpm	Revolution per minute
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification Fermentation
TSEC	Total Sago Effluent Concentrate

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The transportation sector is currently responsible for 60% of global oil consumption (IEA, 2008). It is projected that demands will escalate in the next few decades which may cause disturbances in world-wide supplies and prices. Therefore, concerted efforts are currently ongoing for the development and utilisation of more supply friendly biofuel alternatives (Chin & H'ng, 2013; Tan et al., 2018). Biofuels are fuels whose energy is derived from agricultural and biomass sources (Balat, 2011). Biofuels are categorized as liquid and gaseous fuels that can be produced from numerous sources such as lignocellulosic biomass, agricultural residues and municipal waste. Examples of biofuels include bioethanol, biomethanol, biodiesel, biogas, biosynthetic gas or bio-syngas, biohydrogen, bio-oil, bio-char, vegetable oil and Fischer-Tropsch liquids (Demirbas, 2009). According to Hung et al. (2018), the utilisation of biofuel provides many advantages, which include the reduction of greenhouse gases effects, regional development, job opportunities and energy security.

Currently, the most promising biofuel is bioethanol. Bioethanol is a renewable and sustainable eco-friendly liquid biofuel which is considered to be a promising alternative to fossil fuels (Vincent et al., 2015). In comparison to conventional petrol gasoline, bioethanol produces less than 68% of equivalent energy. However, bioethanol produces cleaner and complete combustion, with water and carbon dioxide the only by-products (Altintas et al., 2002; Yamashita et al., 2010; Vincent et al., 2015).

Bioethanol is mostly produced via the fermentation of starch and carbohydrate agro-industrial products (Vincent et al., 2014). Due to high worldwide demands for bioethanol, the utilisation of greater sources of carbohydrates such as lignocellulosic biomass was introduced (Awg-Adeni et al., 2010; Zakaria et al., 2013). These raw materials are highly preferred as they are abundant and can be acquired at relatively low costs. The usage of lignocellulosic biomass is usually dependant on geographical factors. For example, in the United States utilise corn as the feedstock to produce bioethanol whereas bioethanol production in Brazil is from sugar cane. The total productions of both countries are estimated around 27.6 to 57.7 billion liters in 2016 respectively (Edeh, 2020). Globally, Asian countries such as Japan, China, India and Thailand produce bioethanol from agricultural wastes such as grass, bagasse and wheat straws (Limayem & Ricke, 2012; Mohanty & Abdullahi, 2016). In Malaysia especially in Sarawak (Borneo), sago

(*Metroxylon sago*) is seen as a potential feedstock for local bioethanol production. Sarawak produces about 200,000 tons of sago annually and exports about 47,000 tons to Peninsular Malaysia, Japan, Taiwan and Australia (Ahmad et al., 2022). In 2017, the Premier of Sarawak, Datuk Patinggi Abang Haji Abdul Rahman Zohari Tun Abang Haji Openg had highlighted that Sarawak is currently the largest sago exporter worldwide with the export value of RM 86.8 million to various destination such as Australia, Japan and Taiwan (Sulaiman et al., 2021; Mohamad et al., 2024).

M. sago or sago palm belongs to the *Palmae* family. This particular plant is reported to harboured high quantity of starch within its biomass (Awg-Adeni et al., 2010). Sago starch manufacturing from *M. sago* is a very resource intensive process that require excessive quantities of water during the normal procedure of debarking and processing of sago trunk. A small sago starch processing plant may generate as much as 500 tonnes of sago effluent that are high in fibre and starch content (Bujang, 2006; Anbukumar et al., 2014). According to Awg-Adeni et al. (2010), solid residue by-product such as sago hampas, is usually discharged into nearby stream along with the sago effluent. This practice causes serious water pollution in the surrounding environment due to its excessive contents of biological oxygen demand (BOD) and chemical oxygen demand (COD).

Sago hampas consists of 58% starch, 23% cellulose, 9.2% hemicellulose and 4% lignin (Linggang et al., 2012). The high starch and cellulose contents can be exploited to produce ethanol (Awg-Adeni et al., 2010). According to the study by Bujang et al. (2006), the fibres in sago effluent can be processed to generate an estimated 20% sugars while the sago starch can yield about 98% sugar upon recovery. Hence, there is potential for the production bioethanol from sago wastes.

Total Sago Effluent Concentrate (TSEC) is from the total sago effluent, the solid and liquid residues from the sago processing industries. It contained high starch and fibre which are toxic to the environment but, beneficial in bioethanol production. Thus, the TSEC is a high starchy product of pretreated total sago effluent through mechanical pulverisation and centrifugation process. In this study, TSEC was used as feedstock to produce bioethanol using enzymatic hydrolysis (Simultaneous Saccharification and Fermentation, SSF) via fed-batch fermentation in a non-buffered and buffered broth using different strength of citrate buffer at different biomass concentrations.

1.2 Problem Statement

Unethical disposal of sago effluent from sago processing plant directly into the surrounding ecosystem can lead to serious environmental pollution as sago effluent have

been shown to contain high organic material which could negatively impact the aquatic environment and human health. Economically, the installation of pollution control devices will take up 20% to 50% of the total investment cost of large-scale factory. Thus, numerous studies have been done to overcome this problem. According to previous studies, sago effluent is high in lignocellulosic materials contents which can be used as essential feedstock for bioethanol production. However, bioethanol from lignocellulosic feedstocks requires more complex processing which contribute to the overall production costs. Therefore, a simple and cost-efficient process is needed to utilise the lignocellulosic properties of sago hampas for enhanced bioethanol production.

1.3 Objectives of the Study

The main objective of this research was to produce bioethanol from Total Sago Effluent Concentrate (TSEC) via fed-batch Simultaneous Saccharification and Fermentation (SSF). To achieve this objective, this research was divided into these specific objectives:

- i. To compare the efficiency of bioethanol production from TSEC via fed-batch SSF in a buffered and non-buffered broth.

- ii. To determine the effects of different buffer strengths on bioethanol production from TSEC via fed-batch SSF.
- iii. To determine the effects of different biomass concentration on bioethanol production from TSEC via fed-batch SSF.

1.4 Organisation of the Thesis

This thesis comprises of six (6) chapters. Chapter 1, the General Introduction discusses the overview of the research, the problem statement and the objectives of the study. Chapter 2 is the Literature Review of related topics and findings from previous studies in the same field. This is followed by a preface to bioethanol, the generations of bioethanol production and the perspective of Malaysia in bioethanol production. The discussions flow into details on the process of production of bioethanol from lignocellulosic matters with its components and contributions towards ethanol. Lastly, the chapter concludes on the microorganism involved in this research, *S. cerevisiae*, the process of simultaneous saccharification and fermentation (SSF) and the analyses done for bioethanol production.

Chapter 3 is Materials and Methodology, in which all the materials used in this research are listed in detail. In this chapter, all methods that are employed throughout this

study are stated specifically. Generally, this chapter includes a flowchart of the experimental works which includes all the objectives. Chapter 4 represents the results collected throughout the research. These results include starch content of lignocellulosic matters, HPLC analysis and total carbohydrate analysis using Phenol-sulfuric Assay method. The results are graphically presented and statistical analysis was done using SPSS.

Chapter 5 of this thesis is the Discussion chapter which provides specific justifications based on the results obtained. Chapter 6, the final chapter of the thesis, delivers the summary of the research with general conclusions and some recommendations for future works. Figures and tables for the research are inserted within the main texts on every chapter. All cited literature is listed at the end of the thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Ethanol and Bioethanol

Bioethanol or ethyl alcohol is a flammable and colourless liquid, a promising substitute to fossil fuels (Zabed et al., 2017; Devi et al., 2022). It is a chemical compound consisted of carbon, hydrogen, oxygen and hydroxyl group attached to alkyl radical (Brexó & Sant'Ana, 2017). Bioethanol can be produced from agricultural sources rich in carbohydrates through fermentation by microorganisms such as bacteria or yeast which can be utilised directly as absolute ethanol or mixed with gasoline to form gasohol (Staniszewski et al., 2007; Chin & H'ng, 2013; Azhar et al., 2017). Fermentation by yeast such as *Saccharomyces cerevisiae* is a process whereby sugar (glucose, fructose or sucrose) is fermented to produce cellular energy in the form of Adenosine triphosphate (ATP), ethanol, and carbon dioxide (Huang & Tang, 2007). The production of bioethanol has advanced significantly in countries such the USA, Brazil, Canada, China and several EU nations in attempts to minimise dependence on fossil fuels. Global demands for bioethanol increase over time from 25,700 million gallons in 2015 to 26,059 million gallons in 2020 and possibly due to pandemic, the production of bioethanol declined around 10% in 2019 (Zabed et al., 2017; Devi et al., 2022).

Ethanol, also chemically known as EtOH or C₂H₅OH₂ has the properties of higher-octane levels, higher flammability thresholds, similar or lower flammability rates and higher vaporization heats than gasoline fuel (Table 2.1).

Table 2.1: The fuel properties of ethanol and gasoline (Kumar et al., 2010).

Characteristics	Gasoline	Ethanol
Chemistry	Mixture of hydrocarbons (C ₅ -C ₁₂)	C ₂ H ₅ OH
Specific gravity at 15.55 °C	0.72-0.75	0.79
Distillation temperature (°C)	32-210	78.4
Flash point (°C)	13	12
Kinematic viscosity (mm ² /s)	0.6	1.5
Reid vapor pressure at 37.8 °C(kPa)	35-60	17
Octane number		
a. Research	91-100	111
b. Motor	82-92	92
Oxygen content (wt. %)	0	34.7
Stoichiometric air/fuel ratio (w/w)	14.6	8.97
Net heat of combustion in		
a. MJ/dm ³	32.3	21.2
b. MJ/kg	43.5	27
Heat of vaporization in		
a. MJ/dm ³	0.25	0.66
b. kJ/kg	400	900
Water solubility	0	∞
Vapor flammability limit (vol. %)	0.6-8	3.5-15
Colour	Colourless to light amber	Colourless

	glass	
Vapor toxicity	Moderate irritant	Toxic in large doses
Flame temperature at 101.325 kPa (°C)	392	478
Maximum flame speed (m/s)	0.40	0.33

Therefore, the usage of bioethanol is broadened as gasoline improver or octane booster as well as the combination of bioethanol with diesel promotes the reduction of exhaust gasses emission (Nigam & Singh, 2011). Moreover, in comparison to fossil fuel, bioethanol has numerous advantages as bioethanol is readily biodegradable and environmentally friendly as it releases safe amounts of carbon dioxide which make it less toxic and contribute to reduction of air pollution (Kheshgi et al., 2000; John et al., 2011). These fuel characteristics enable internal combustion engines to operate more efficiently than gasoline by enabling higher compression ratios, shorter burn times, and leaner engines (Balat & Balat, 2009). Aside from being used as power sources of automobiles, bioethanol is also used widely in the industries of chemicals, pharmaceuticals, cosmetics, medical and beverages (Chin & H'ng, 2013).

At present, four generation of designated bioethanol process known as first (1G), second (2G), third (3G) and fourth (4G) as shown in Figure 2.1. The first generation bioethanol involves the pre-processing of food-based feedstocks notably sugarcane, corn

and wheat (Eardley & Timson, 2020). Brazil and the United States (US) started the mass production of bioethanol in the 1970's from feedstock rich in sucrose such as corn and sugarcane. Globally, Brazil and the US are the major producers of ethanol which account for about 35% of world production (Chin & H'ng, 2013). Nevertheless, high demand for bioethanol production had once caused a sudden increase of global food prices. Current interests are in production of second generation bioethanol derived from lignocellulosic biomass (wood, grasses & straw) and third generation bioethanol derived from algal biomass which includes microalgae and macroalgae (Harun et al., 2009; Nigam & Singh, 2011; Chin & H'ng, 2013). According to Nair et al. (2022), the fourth generation bioethanol is known as an advanced version third generation bioethanol whereby the production is from genetically modified organisms (GMO). While more studies have focused on second generation bioethanol since 2010 whilst third generation bioethanol is still under investigation, Kang et al. (2014) had provided a complete discussion of the first generation bioethanol process. Nevertheless, the production of ethanol through either first, second or third generation feedstock are useless in industrial and fuel application since this process produces alcohol-lean broth only thus, must be purified via fractional distillation (Kang et al., 2014).

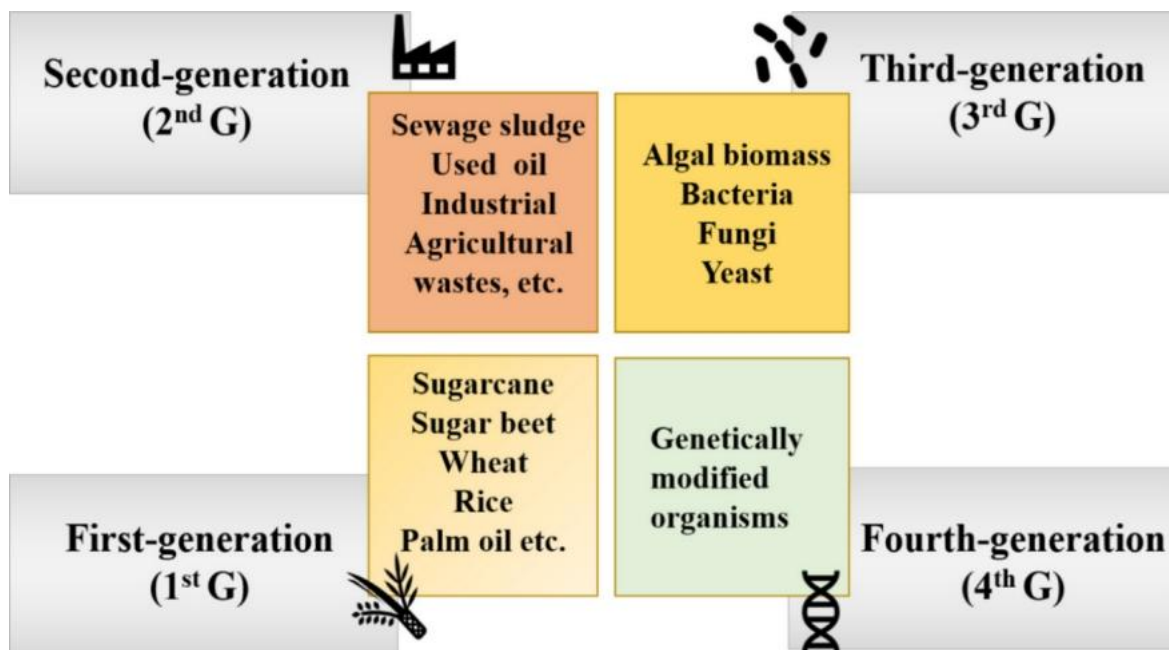


Figure 2.1: Simplified illustration of different generations of bioethanol production (Nair et al., 2022).

2.1.1 First Generation Bioethanol

Bioethanol production from sugar-based raw materials is known as the first generation (1G). The 1G bioethanol is commonly produced from food-based crops such as wheat, sugarcane, sugar beet, rice and soybean oil (Zabed et al., 2017; Nair et al., 2022). Commercially, the 1G bioethanol is widely practiced in fuel and ethanol industries of many countries. Corn is commonly used feedstock in the United States, while Brazil used sugarcane. In Europe, both feedstocks with high levels of starch and sugar (potatoes, wheat and sugar beets) are commonly used in the ethanol industry (Tse et al., 2021). The conventional methods utilised only the germs from the seeds or grains to hydrolyse starch

into glucose which then fermented by yeast into ethanol. Lastly, distillation and dehydration are conducted to obtain desired concentration of ethanol, as shown in Figure 2.2 that can be blended with fossil fuels or utilised directly as fuel (Robak & Balcerek, 2018). The 1G bioethanol is quite controversial however, due to food-feed-fuel competition by which concerns related with availability of croplands and food security for both human and livestock (Srivastava et al., 2020; Tse et al., 2021). These issues may lead to disruption of food chain due to resource depletion and soaring price of food commodities (Robak & Balcerek, 2018). Nevertheless, the 1G bioethanol continues to produce more than 95% of the 109 billion liters of ethanol available worldwide in 2019 and provides relief in greenhouse gas (GHG) emission (Srivastava et al., 2020; Bertrand & Dussap, 2022).

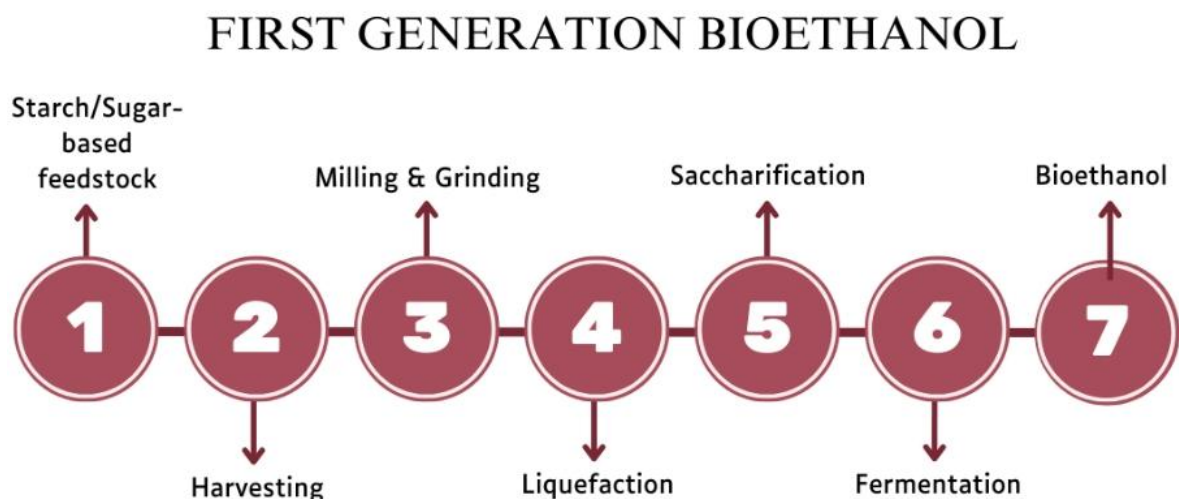


Figure 2.2: Flow chart of the first generation bioethanol (adapted from Dutta et al., 2014).

2.1.2 Second Generation Bioethanol

The bioenergy competitions have led to a finding of an alternative which referred as the second generation (2G) bioethanol (Srivastava et al., 2020). Figure 2.3 shows bioethanol from second generation is produced from non-edible renewable sources such as sewage, municipal solid waste (MSW), agricultural and industrial waste. The 2G bioethanol is considered a feasible option as there are abundant of lignocellulosic biomass worldwide to be used as feedstocks without any controversial disputes (Srivastava et al., 2020; Nair et al., 2022). Although the use of these feedstocks are relatively inexpensive and readily available for bioethanol production, the 2G bioethanol require more advanced technologies to process the conversion to reducing sugar prior to fermentation. The lignocelluloses are mainly composed of cellulose, hemicellulose and lignin which form complex structures that are more resistant to chemical or biological breakdown compared to starch materials (Robak & Balcerek, 2018; Tse et al., 2021). Thus, pretreatment of these lignocellulose structures are necessary for sugars in cellulose and hemicellulose able to be converted to bioethanol through fermentation. The pretreatments differ as the lignocellulose contents vary among feedstock (Tse et al., 2021). According to Edeh (2020), the 2G bioethanol has greater potential to produce more bioethanol as well as reducing the GHG emission compared to 1G bioethanol.

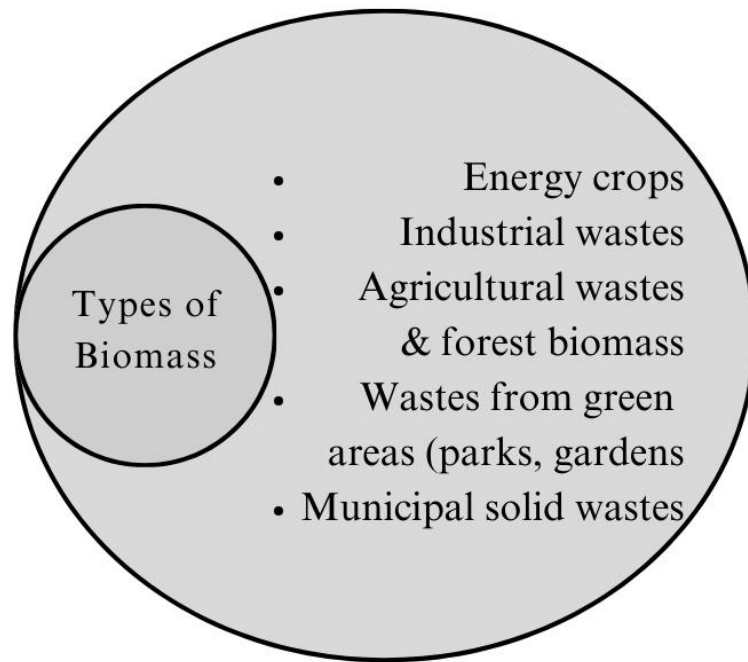


Figure 2.3: Types of second generation biomass (Sims et al., 2010).

2.1.3 Third Generation Bioethanol

The third generation (3G) bioethanol is produced from algal biomass, a cheaper and readily available feedstock. These algae (microalgae & macroalgae) are simple chlorophyll-containing photosynthetic organism (Srivastava et al., 2020). The 3G bioethanol is regarded to be a substitute to 1G and 2G bioethanol due to its high productivity, rapid absorption of carbon, easy cultivation and requires less land (Tse et al., 2021). The 3G bioethanol also gained worldwide interest due to its high concentration carbohydrates and absence of lignin component in the algae structure (Edeh, 2020). Moreover, the production process of 3G bioethanol is expected to be cost-efficient as

lignin removal process is eliminated and minimised industrial waste streams (Robak & Balcerek, 2018; Edeh, 2020; Hebbale & Ramachandra, 2022). Nevertheless, pretreatment process is essential for 3G bioethanol to disrupt algal cell wall prior to saccharification process. It is a conversion process of complex carbohydrates to sugar by enzymatic hydrolysis and followed by ethanolic fermentation as shown in Figure 2.4 (Dave et al., 2019; Tse et al., 2021). The pretreatment is the most crucial step as it varies depending on algal species. Inadequate pretreatment could result in inefficient saccharification process, formation of by-products (acetic acid & formic acid) and affect bioethanol production (Dave et al., 2019; Tse et al., 2021). Furthermore, this generation of bioethanol is still in preliminary stage limited at laboratory scale compared to other feedstocks which already shown potential on commercial scale (Zabed et al., 2017). Despite the complexity of the process, Robak & Balcerek (2018) and Tse et al. (2021) reported that the ethanol yield from algal biomass are changeable and some produce low ethanol yield compared to 1G and 2G bioethanol. The 3G bioethanol has shown greater potential as feedstock and continue to grow through advancement of current technologies for bioethanol production in the future (Dave et al., 2019; Tse et al., 2021).

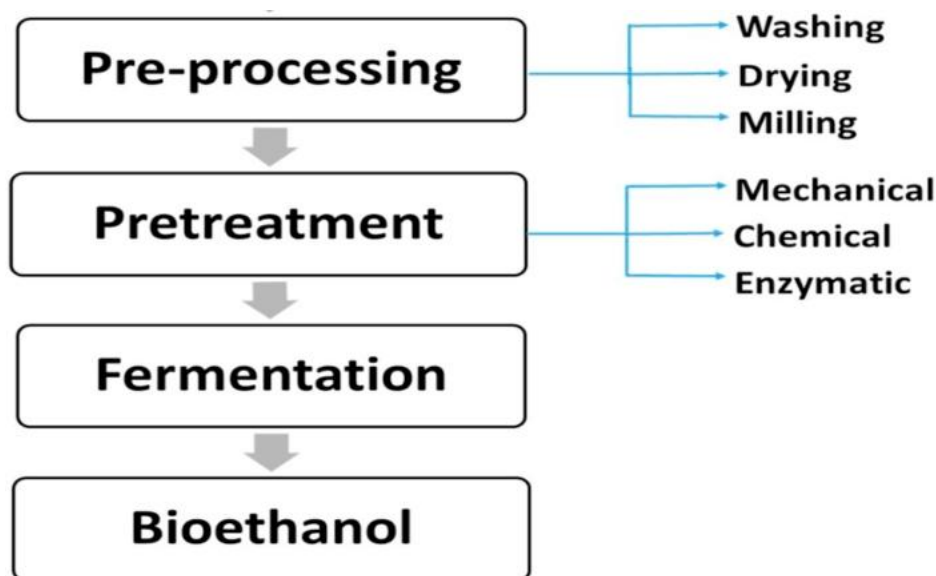


Figure 2.4: Flowchart of the third generation bioethanol (Dave et al., 2019).

2.1.4 Fourth Generation Bioethanol

The latest technology of bioethanol production is termed as the fourth generation (4G) bioethanol. It is an advanced version of 3G bioethanol which produced from the combination of genetically engineered microbes and feedstocks (Srivastava et al., 2020; Tse et al., 2021; Cavelius et al., 2023). According to Figure 2.5, the 4G used bioengineering techniques to modify the feedstocks and enhance carbon fixation which can be stored during production process (Ale et al., 2019; Srivastava et al., 2020). Such advancements allow the feedstocks to absorb broader range of light spectrum and capture more carbon dioxide emissions during production stages. A wide range of techniques for model organism such as *Escherichia coli* and *S. cerevisiae* are also reported by Cavelius et al. (2023) which include genetic modification of the metabolic pathway or addition of new

pathways in the native producers to enhance bioethanol production. The production 4G bioethanol only requires minimum steps of converting energy provided from the sun into bioethanol; costly fermentation and processing steps are not required (Ale et al., 2019; Srivastava et al., 2020). Regardless of the advantages, the 4G bioethanol have several backtracks which include ethical issue regarding genetically modified organism (GMO) as well as limited bioengineering technology. Moreover, the carbon fixation techniques are restricted in laboratory scale therefore, insufficient for further application of this feedstock (Srivastava et al., 2020). Thus, with following alteration, the 4G bioethanol can be the next potential alternative in bioethanol industry from the global perspective and towards greener and eco-friendly environment in the future.

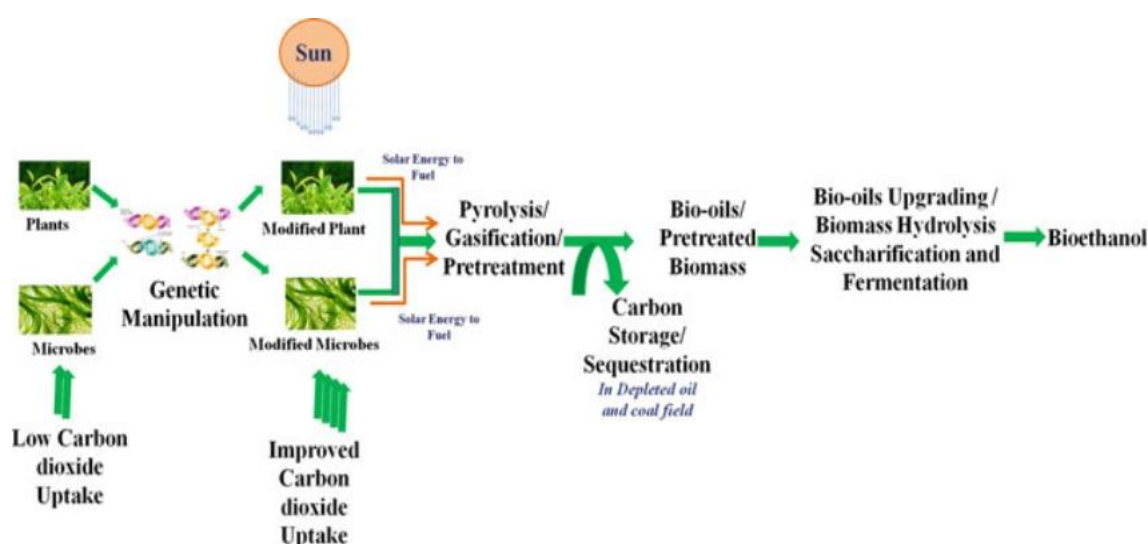


Figure 2.5: Schematic illustration of the fourth generation bioethanol production (Srivastava et al., 2020).

2.2 Bioethanol in Malaysia

Consumption of fossil fuels is estimated at about 80% primary energy worldwide in 2019 where 60% were utilised by the transportation sector (Halдар & Purkait, 2020). The demand of biofuels has increased worldwide as alternative fuels (Rezania et al., 2020). Tropical countries such as Malaysia have wide cultivable natural land for biomass production which is the main resources for biofuels production. Biofuels are categorized as liquid (biodiesel & bioethanol) and gaseous (biogas & biohydrogen) that can be produced from numerous sources such as lignocellulosic biomass, municipal waste and palm oil residues. In accordance with the Paris agreement signed by Malaysia in 2016, Malaysia had planned to reduce the emission of its greenhouse gas (GHG) by 45% by 2030 (Rezania et al., 2020). The National Biofuels Policy formulated by Malaysia was implemented in March 2006 with objectives to set biofuel with other energy sources while preserving the nation's environment and boosting its prosperity (Chin & H'ng, 2013).

According to Rezania et al. in 2020, difficulties such as 1) costly transportation budget from countryside to urban processing factories 2) excessive capital expenditure and 3) insufficiency of high technology, despite having abundance of natural sources and biomass waste. Besides, documentations from licensed government agencies are needed to obtain and transport these biomass materials (Goh et al., 2010). Hence, the technology of

bioethanol production in Malaysia has yet to be implemented and is continuously revised for significant growth. According to Tan et al. (2008) and Goh et al. (2010), few fundamental strategies are outlined by the Malaysian government with the aim of promoting second generation bioethanol as an alternative fuel such as 1) financial support from government or private sector for researchers; 2) offers of suitable loans for producers and incentives for per litre of bioethanol produced; and 3) cooperation of both government and private sectors for systematic Standard Operation Procedure (SOP). The Government of Malaysia also proposed a mandate to blend gasoline with bioethanol as the utilisation of oil palm biomass for bioethanol production is predicted to significantly boost the economic sector (Chin & H'ng, 2013; Rezania et al., 2020). However, bioethanol as replacement for fossil fuels in Malaysia has been reported as slow progression due to shortage of feedstock, plantation area and overall costs. Moreover, Malaysia is still new in the bioethanol industry and potential bioethanol production has yet to be commercialized (Kumar et al., 2013; Rashidi et al., 2022). To date, only Malaysian palm biodiesel had been accepted by the Japan Automobile Manufacturers Association (JAMA) for transportation consequently, making Malaysia as the primary biodiesel producer aside from other developing countries such as the USA, France and Brazil (Rashidi et al., 2022).

2.3 Bioethanol from Lignocellulosic Biomass

Biomass is generally referred to as renewable organic compounds produced by plants via photosynthesis to form oxygen, carbohydrates and energy rich organic matter. Lignocellulosic biomass is a main renewable resource which the structural component of woody and non-woody plants (Allen, 2016). Available biomass can be divided into few groups that include primary source, secondary sources and tertiary sources. For example, primary sources include sugar cane, perennial grasses and short rotation energy plantations which are either produced as crop or key product whilst secondary sources are agricultural residues from the production processes such as rice husks, bagasse and straw. Lastly, tertiary sources are organic fractions of municipal solid wastes (MSW), residues produced during and after application ends (Fischer & Schrattenholzer, 2001; Kumar et al., 2009; Limayem & Ricke, 2012). In Figure 2.6, the lignocellulosic biomass is composed of three main components; cellulose (30 – 50%), hemicellulose (15 – 35%) and lignin (5 – 30%). Cellulose is an important constituent of lignocellulosic biomass and makes up approximately 70% of the entire biomass in combination with hemicellulose with the remaining components including ash and others (Balat, 2011; Limayem & Ricke, 2012; Allen, 2016). Thus, the efficiency of bioethanol production is dependent on the

composition of lignocellulosic biomass as it is highly variable due to environmental and genetic factor (Vohra et al., 2014).

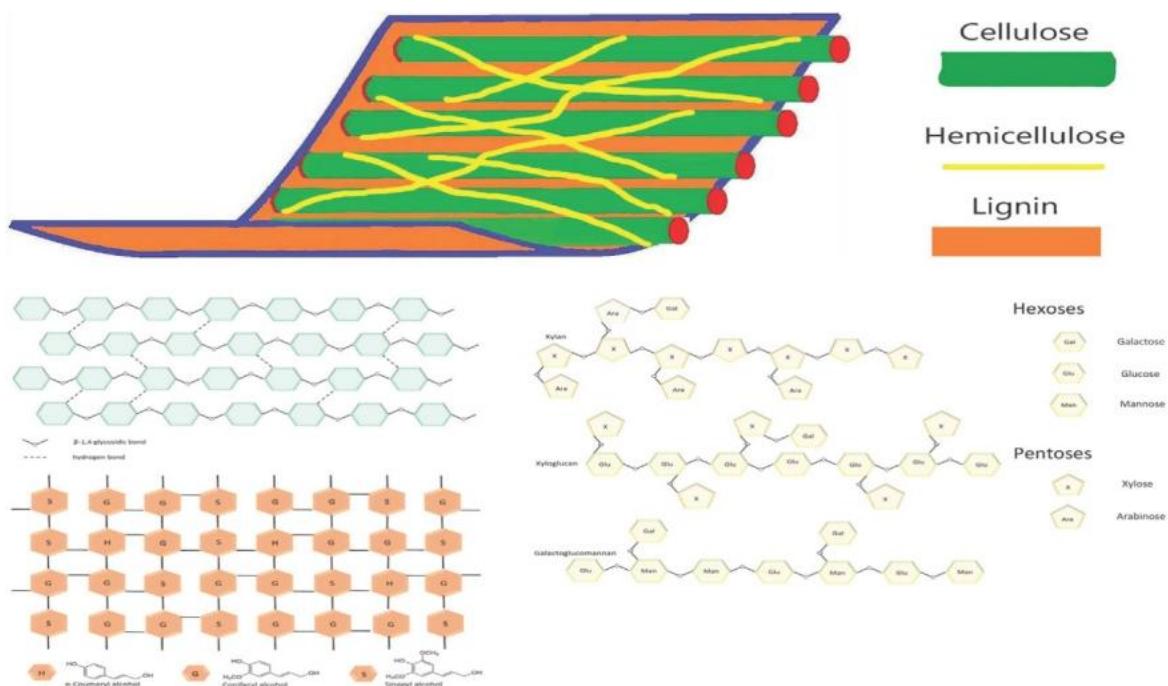


Figure 2.6: Illustration diagram of lignocellulosic structure (Yankov, 2022).

Notably, lignocellulosic materials are the cheapest feedstock for the production of bioethanol as it is abundantly obtained from agricultural residues, forestry biomass and municipal waste (Goh et al., 2010). In 2019, Malaysia was the second-largest oil palm producer worldwide exceeding 20 million tonnes and correspondingly producing massive amounts of biomass which are forecasted to reach about 100 million tonnes by 2020 (Chin & H'ng, 2013; Rezania et al., 2020). Table 2.2 illustrates the recent area of oil palm plantations in Malaysia by state.

Table 2.2: The oil palm plantation area in Malaysia (Parveez et al., 2023).

State	Oil Palm Plantation Area (Million Hectares/Year)	
	2021	2022
Sarawak	1.61	1.62
Sabah	1.52	1.51
Peninsular Malaysia	2.61	2.54
Total	5.74	5.67

Other biomass materials also produced per annum include rice husks, wood waste, timber and coconut trunk fibres. However, the wastewater of oil palm contains high amounts of organic substances which can cause environmental issues if discharged into nature (Kamyab et al., 2018). Thus, the consumption of these waste materials could overcome water pollution from the disposal and minimise the expenditure of waste treatment.

According to Chin & H'ng in 2013, biofuels technologies can be classified by the terms conventional and advanced through which first generation bioethanol is categorized as conventional and bioethanol from biomass or waste materials is categorized as advanced. Presently, more interest in the production of bioethanol from agricultural waste prior to its abundant availability and economical potential feedstock which does not interrupt food supplies. However, advanced biofuels technology or also referred to as second and third generation are still either in the research and development (R&D), demonstration or pilot study on converting lignocellulosic biomass into bioethanol. Researchers at present are developing methods to increase yield of cellulosic ethanol (Chin & H'ng 2013). The complex structure of lignocellulosic biomass requires complicated procedures which

includes pre-treatment, hydrolysis, fermentation, distillation and lastly, recovery of ethanol (Rezania et al., 2020). Figure 2.7 shows the process of the lignocellulosic bioethanol production.

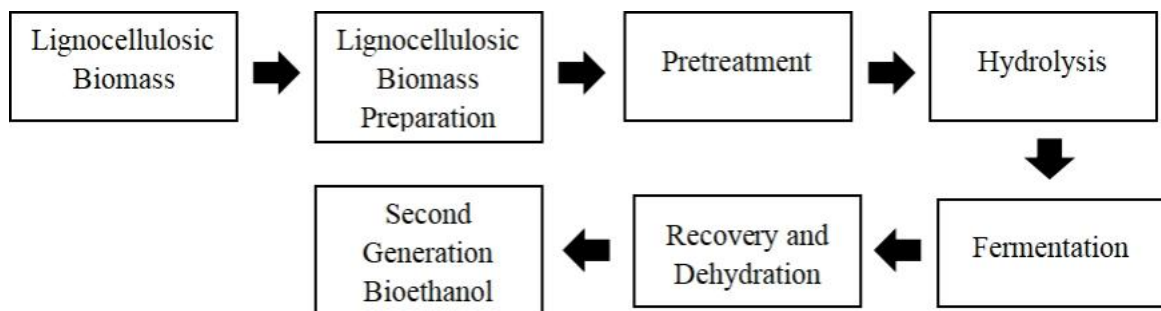


Figure 2.7: Process of the lignocellulosic bioethanol production (Rezania et al., 2020).

Primarily, pretreatment is a procedure done to remove the outermost seal of lignin and exposing carbohydrates (cellulose and hemicellulose) to the hydrolysis process which is known to consume huge expenditure (Halдар & Purkait, 2020). The biochemical conversion of lignocellulosic biomass has certain technical challenges due to its heterogeneous structure which requires the application of various pre-treatment and/or alteration in raw materials (Alexis & Arturo, 2018). Therefore, improved and more cost-effective pre-treatment procedures, as well as strategies to minimise or remove microbial and chemical pollutants that might affect yields, require additional research. It was reported that microfiltration (suspended solids), ultrafiltration, nanofiltration and reverse

osmosis handling the dissolved pollutants were helpful in the resolution of some of the problem (Kang et al., 2014).

On the other hand, enzymatic hydrolysis is a process of breaking down starch from biomass to monosugars with the aid of acids (diluted or concentrated) and/or enzymes. Presently used as feedstock of bioethanol production, the conversion of lignocellulosic biomass to monosaccharides is more complex than the hydrolysis of starch (Chin & H'ng, 2013). Lignin is the third main constituent of lignocellulosic biomass along with cellulose and hemicellulose, consisting of hydrophobic surface which limits the ability of enzymes to bind and hydrolyse cellulose to glucose (Huron et al., 2016; Alexis & Arturo, 2018). Several approaches had been designed to reduce extra costs related to catalysts and genetic modification technology to increase ethanol yield whilst reducing inhibition of end-product (Halder & Purkait, 2020). Thus, the development of simultaneous saccharification and fermentation (SSF) and combined fermentation (SSCombF) in a single reactor had demonstrated the ability to avoid product inhibition and increase product yield in a short period of time (Kang et al., 2014; Halder & Purkait, 2020).

Fermentation is the biological process by which a variety of microorganisms, such as bacteria, yeast, or fungi, metabolize hexoses and pentose into ethanol. According to

Fischer & Schrattenholzer (2001) and Kang et al. (2014), the chemical pathway for monosaccharides (C5 and C6) is shown in Figure 2.8.

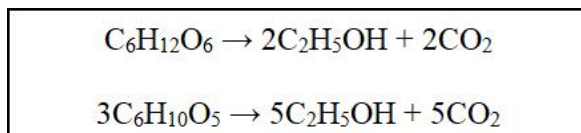


Figure 2.8: The chemical pathway of monosaccharides (C5 and C6) (Fischer & Schrattenholzer, 2001; Kang et al., 2014)

Despite its low ethanol yield and inability to metabolise xylose, *Saccharomyces cerevisiae* is the preferred yeast for ethanol production. Other species of *Saccharomyces* such as the natural wild type shows capability to replace *S. cerevisiae*. However, it yields lower ethanol and is vulnerable to stress conditions. Xylose-fermenting yeast such as *Candida*, *Dekkera* and *Pichia* nonetheless, produce low ethanol concentrations and acetic acid, a fermenting-yeast inhibitor (Kang et al., 2014). The development of genomic technologies is a powerful indicator to develop novel techniques to increase ethanol fermentation performance. The research of recombinant DNA technology can be used to upregulate stress tolerance genes, which can be a helpful tool to overcome inhibitory conditions (Dogan et al., 2014). However, the usage of genetically modified microorganisms in large-scale industrial fermentation had gained concerns to community

health prior to the development of antibiotic-resistance strains which can potentially harm environment and community health (Kang et al., 2014).

The researched lignocellulosic biomass produces more bioethanol per tonne feedstock (L/t) than the majority of marketed bioethanol feedstock. High amounts of glucose convertible substance as potential feedstock had shown a major impact on the yield of ethanol (Chin & H'ng, 2013). In order to achieve high conversion and cost-effective biomass into bioethanol operation, improvements are needed on both these aspects (Kang et al., 2014). Overall, the obstacles posed by bioethanol are enormous, but given the rapid advancements of this field, it is certain that interesting developments will occur (Hahn-Hagerdal et al., 2008). According to Rashidi et al. (2022), the bioethanol industry in Malaysia is relatively new and lack in terms of experiences, technologies and plantation area of feedstock. Therefore, rather than as producer, Malaysia is currently amongst the major importer of bioethanol products which predicted to produce about 7.2 billion litres of bioethanol by 2023.

2.3.1 Cellulose

Lignocellulosic biomass contains 40-60% cellulose on a dry weight basis (Ragauskas et al., 2014). It is a linear of polysaccharides which made of β -D-

glucopyranose units connected by β -1,4 glycosidic bonds as shown in Figure 2.9 and cellobiose as the primary repeating unit (Zoghلامي & Paes, 2019; Yankov, 2022). According to Awg-Adeni in 2010, cellulose fraction was composed primarily about 89% of glucose and small amounts of other sugars such as arabinose, fructose, galactose, mannose and xylose. Cellulose fibrils, made of strong hydrogen bonds and van der Waals forces showed crystalline nature that makes it recalcitrant to enzymatic hydrolysis (Robak & Balcerek, 2020; Zoghلامي & Paes, 2019).

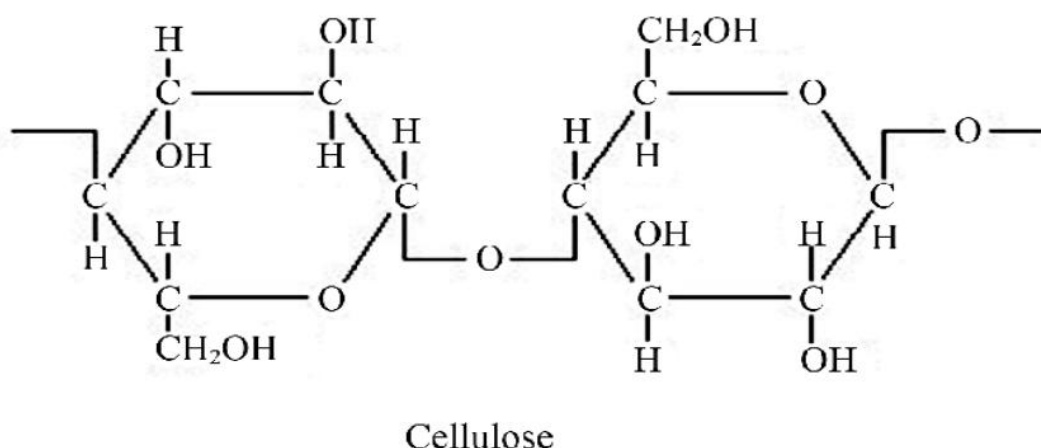


Figure 2.9: The chemical structure of cellulose (Richards et al., 2012).

2.3.2 Hemicellulose

Hemicellulose is heterogeneous groups of lignocellulosic biomass that contain monosaccharides subunits, representing 20-35% of the biomass weight. Figure 2.10 shows

hemicellulose is made of short branched polymer formed by various of monosaccharides which are pentoses (arabinose & xylose), hexoses (galactose, glucose & mannose) and uronic acid (Balat, 2011; Robak & Balcerek, 2020; Yankov, 2022). These monosaccharides made up a linear chain of xylans and other branch chain such xyloglucans, mannans and glucomannans (Robak & Balcerek, 2020; Guo et al., 2022). Hemicelloses vary among the raw material whereby softwood hemicelluloses are dominantly composed of hexoses and pentoses in hardwood hemicellulose. Its amorphous nature allows the hemicellulose to hydrolyse easily by dilute acids or bases compared to cellulose (Balat, 2011; Zoghلامي & Paes, 2019).

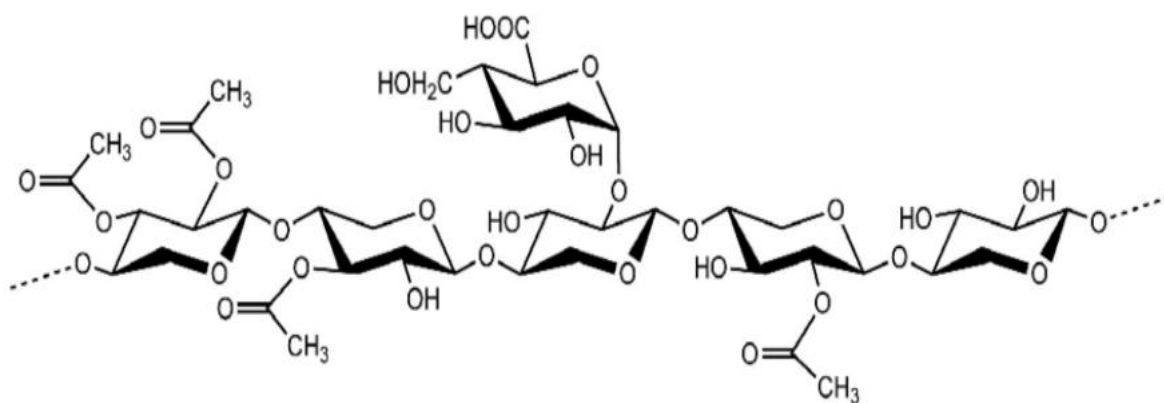


Figure 2.10: The chemical structure of hemicellulose (Hu et al., 2020).

2.3.3 Lignin

On dry basis, lignin comprised about 15-40% of lignocellulose biomass, second most abundant polymer after cellulose. According to Balat (2011) and Guo et al. (2022), lignin is a complex amorphous phenolic polymer made from phenylpropanoid units (coumarin, sinocrocinol & coniferyl alcohol). Figure 2.11 showed the lignin binds cellulose and hemicellulose in the cell wall, liable for maintaining rigidity of structure, resisting pressure as well as a physical barrier during hydrolysis process (Zoghلامي & Paes, 2019; Guo et al., 2022). Similar to hemicellulose, lignin contents differ in each raw material. Lignin can range from 20% to 40% in both softwoods and hardwoods (Balat, 2011; Yankov, 2022). However, high concentration of lignin causes a major limitation to the production of lignocellulosic bioethanol as it causes recalcitrance during enzymatic hydrolysis process by unproductively adsorbing cellulases simultaneously reducing the activity of cellulase (Balat, 2011; Robak & Balcerek, 2020). Thus, the lignin is usually removed during pretreatment process to enhance biomass yield. Recently, pretreatment of lignin have evolved towards genetic modification of the structure to improve conversion of biomass without the necessity of lignin removal (Yoo et al., 2020).

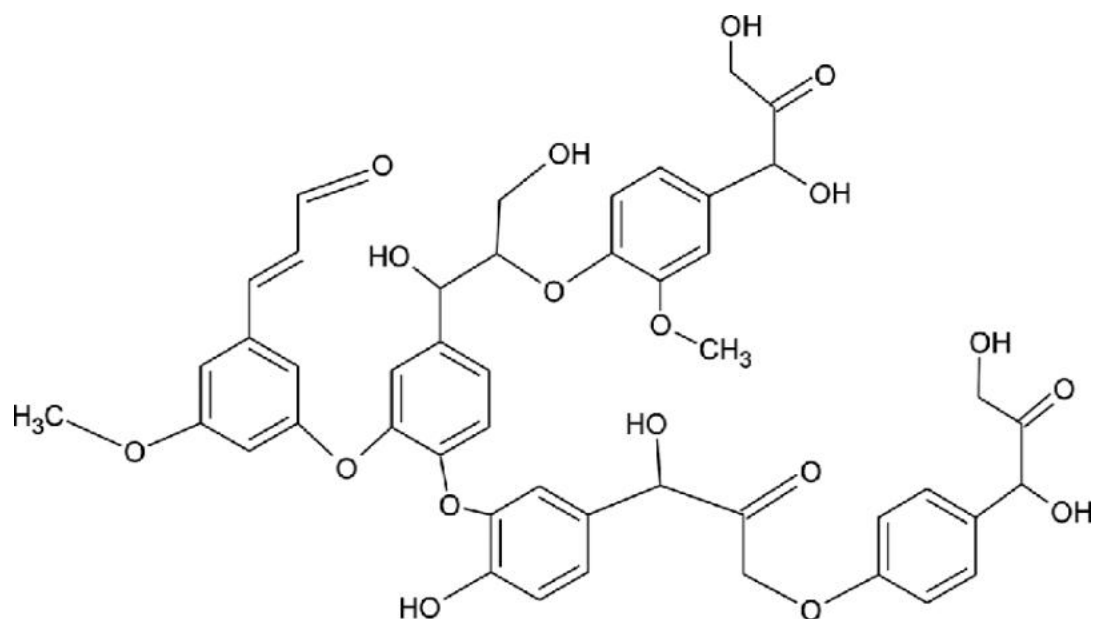


Figure 2.11: The chemical structure of lignin (Mahmood et al., 2018).

2.4 Sago palm

Metroxylon sagu Rotbb. also known as sago palm is a species belonging to the order of *Arecale* and family of *Palmae* which is an important crop for the socioeconomic of Southeast Asia. Sago palm is an essential dietary source for many people whereby the early inhabitants of the Malay Archipelago depended on it as their staple food before the discovery of rice (Abd-Aziz, 2002; Awg-Adeni et al., 2010). According to Bujang (2006), a mature sago palm can grow up to 10-12m high and about 0.8-1m in circumference without the need of fertilization (Figure 2.12). The genus *Metroxylon* is generally hapaxanthic, the flowering only happens once in lifetime, followed by the fruiting process and shortly leads to the death of the palm (Abd-Aziz, 2002; Karim et al., 2008; Lim et al.,

2019). During the vegetation phase which lasts an average of eleven years, nutrients obtained through photosynthesis are converted into starch and fill up the trunk for long-term starch storage (Abd-Aziz, 2002; Lim et al., 2019).



Figure 2.12: Cluster of sago palm at average length of 7 to 15 m (Karim et al., 2008).

According to Lim (1991), in Sarawak, the growth of sago palm is classified into 5 stages; “Plawei” is the maximum vegetative growth of the palms, “Plawei Mani” is the

emergence of inflorescence stage, “Bubul” is the development of inflorescence stage, “Angau Muda” is the flowering stage and “Angau Tua” is the final stage of fruiting. Matured sago palms yield the highest starch per hectare (~ 25 tons), 3 to 4 times more than corn, rice or wheat and 17 times more than cassava (Karim et al., 2008; Amin et al., 2019; Lim et al., 2019). Its large fibrous root is beneficial to the environment by providing protection against pollutants, contaminants, floods and fire drought (Amin et al., 2019). In addition to its natural pests and diseases, the sago palm does not require any fertilisation (Abd-Aziz, 2002). The sago palm ideally grows in humid lowland swamp and tropical forest. It is native species of the Southeast Asia, specifically Papua New Guinea, Malaysia, Indonesia, Thailand and the Philippines (Ehara et al., 2018; Lim et al., 2019).

Malaysia has great potential for starch production from sago palm (Abd-Aziz, 2002). The potential of sago was recognized by the Sarawak government and therefore authorized the Land Custody and Development Authority (LCDA) to launch industrial sago cultivation and implement management strategies. The sago palm plantation was started in 1986 and to date, the largest sago palm plantation areas in Malaysia is located in the Mukah division in Sarawak. Sarawak is globally known as the largest exporter of sago starch to different countries with about 40,000 tonnes per annum and expected to increase in demand (Amin et al., 2019). The sago starch in the form of air-dried flour mainly used

in a variety of dishes such as jellies, puddings, noodles and recently, sago pearls (Abd-Aziz, 2002; Karim et al., 2008).

Generally, sago starch extraction from sago palm is divided as traditional and modern. There are few cycle of this process to extract best quality and quantity of sago starch (Bujang & Ahmad, 2000). In Sarawak, modern technique of extraction is adopted, where most factories are completely mechanized and simultaneously reducing laborious works (Karim et al., 2008; Awg-Adeni et al., 2010). This extraction process includes few stages of debarking, rasping, sieving, settling washing and drying (Figure 2.13). The pith from debarking process is further broken down by a hammer mill resulting in slurry starch. Subsequently, the course fibers are separated using a series of centrifugal sieves. Further purification is carried out by separation in a nozzle separator to obtain pure starches. The final stage of starch dewatering is achieved using rotary drum and hot air drying (Awg-Adeni et al., 2010). This extraction process is known to produce three main types by-products; sago bark, sago hampas and sago effluent.

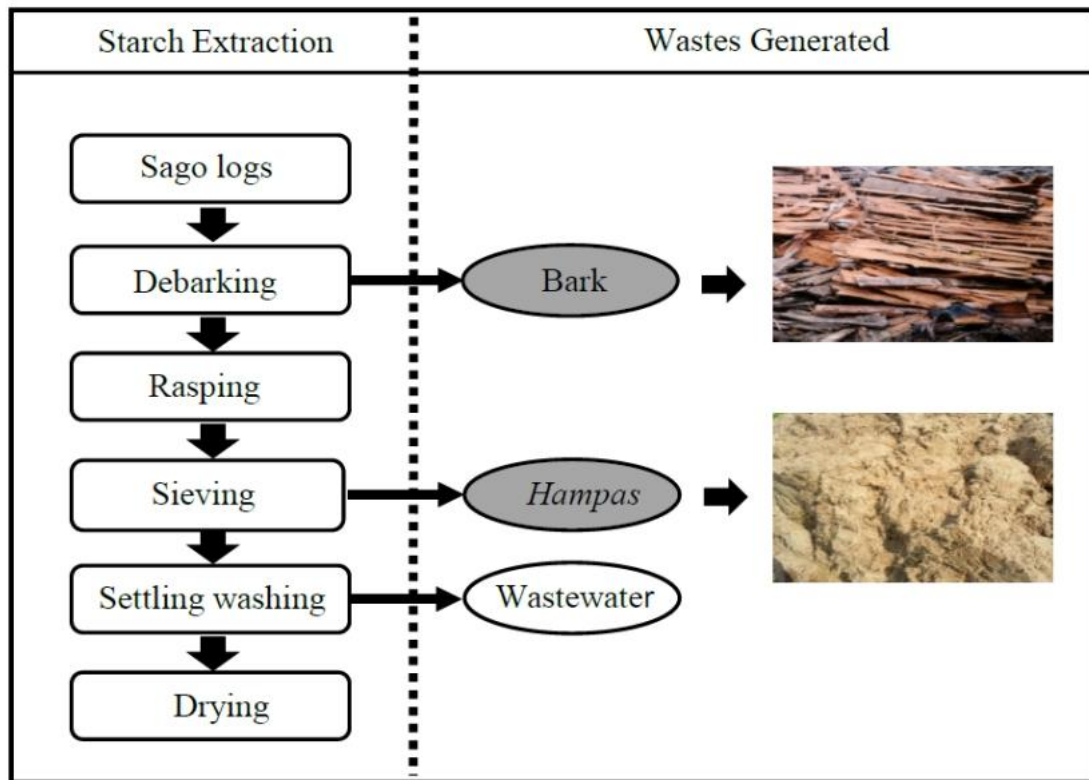


Figure 2.13: Illustration of the sago starch extraction process (Amin et al., 2019).

2.4.1 Sago hampas

Sago hampas is inexpensive starchy residue resulting from *M. sagu* after the sago starch extraction process (Figure 2.14) (Awg-Adeni, 2010). According to Muradi et al. (2020), Sarawak, mainly in Sibul and Mukah divisions had produced approximately 40 tonnes of sago hampas daily and usually discharged into nearby stream. This unethical dumping activities cause elevation of biological oxygen demand (BOD), depletion of oxygen content in the stream which endanger aquatic lives and raising environmental concerns (Amin et al., 2019). The sago hampas are mainly composed of starch, cellulose,

hemicellulose and lignin. On dry basis, sago hampas has about 58% starch, 20.7% cellulose, 11.2 % hemicellulose, and 3.3% lignin (Linggang et al., 2012; Alias et al., 2021). Due to its high polysaccharide and fibrous lignocellulosic contents, sago hampas has been used as feedstuff for swine, mushroom compost and for manufacturing particleboard (Phang et al., 2000). Moreover, Kumaran et al. (1997) had also reported that sago hampas has great potential as cheap carbon source for laccase production and several enzymes via fermentation. More importantly, many researchers had studied and highlighted on the feasibility of sago hampas as feedstock for bioethanol production (Muradi et al., 2020). The sago hampas either in dry or wet form can be converted into glucose through enzymatic hydrolysis prior to fermentation process. It is a cost-effective to utilise the sago hampas as substrate for bioethanol production and at the same time reducing environmental pollution caused by the sago extraction industries (Awg-Adeni et al., 2010; Amin et al., 2019).

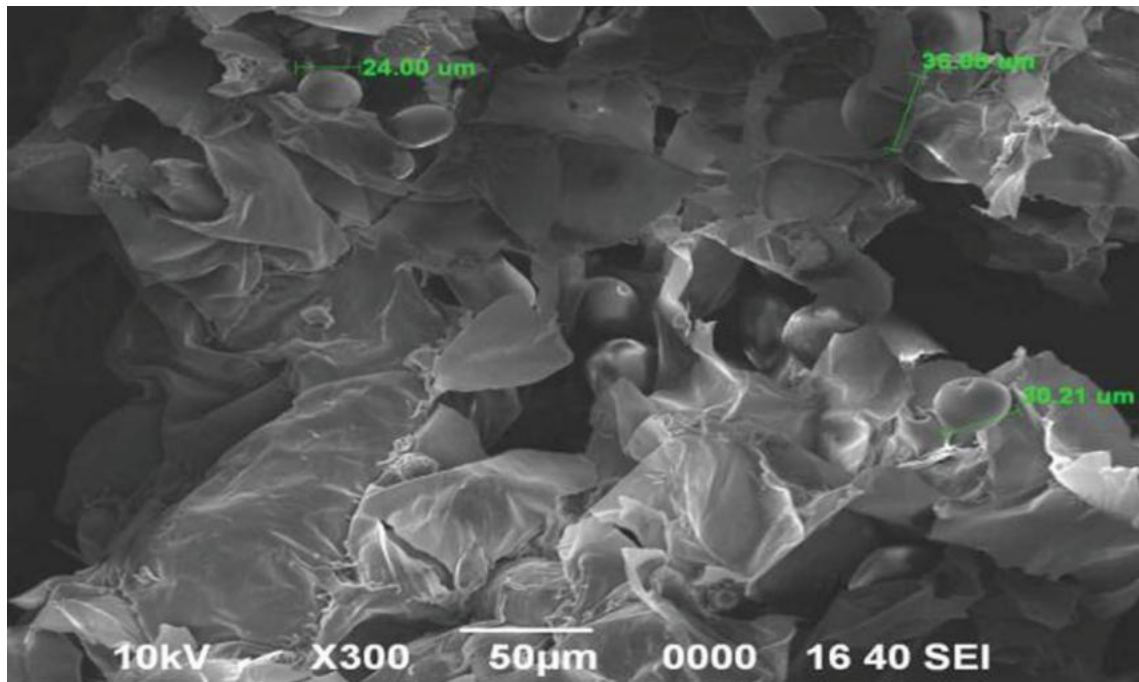


Figure 2.14: Untreated sago hampas under Scanning Electron Microscope (Awg-Adeni et al., 2013).

2.4.2 Sago effluent

The sago effluent is a wastewater from the sago starch production process. Voluminous amount of water are required for the starch extraction process and discharged as sago effluent (Figure 2.15), rich with organic materials (Awg-Adeni et al., 2010; Rashid et al., 2020). The sago effluent comprises about 94-97% of liquid and the remaining of solid waste which must be treated before disposing it into the environment. However, only few sago factories implemented pretreatment systems for environment preservation purposes (Wee et al., 2017). The sago effluent has the potential of causing water pollution

due to its high content of organic matters such as carbohydrates, lipids, nitrogenous compounds and proteins (Awg-Adeni et al., 2010; Santoso, 2018). It is reported by Wee et al. (2017) that the sago effluent is acidic with pH between 3.4 to 4.7 and emits an obnoxious odor thus, pollutes and deteriorates the quality of environment worldwide. Commonly, the sago effluents are either disposed into nearby streams or dumped in factory facilities (Awg-Adeni et al., 2013; Santoso, 2018). Some sago starch industries in Malaysia find an alternative to burn these residues however, open burning of sago contributes to air pollution and wastage of valuable resources. Total production of sago starch and unethical disposal of sago effluent are generated approximately at the same amount thereby causing an alarming environmental concern (Santoso, 2018; Rashid et al., 2019). Moreover, the high concentration chemical oxygen demand (COD), about 5820 mg/L in the sago effluents has violated the regulatory limit discharge established by the Environmental Quality Act of 1974 and also endangered marine life. Thus, many present studies are done with the efforts to exploit these residues into valuable resources simultaneously maintaining the ecological system (Awg-Adeni et al., 2010; Amin et al., 2019).



Figure 2.15: Sago effluent from the sago mills (Vincent et al., 2021).

2.4.3 Total Sago Effluent Concentrate (TSEC)

Total Sago Effluent Concentrate (TSEC) refers to the starchy solid component of the sago effluent obtained through physical pretreatment of mechanical pulverisation and centrifugation. Generally, the sago waste contains high concentration of starch, about 65.7% and utilised as an alternative substrate for glucose production (Rashid et al., 2019). The lignocellulosic components in the TSEC can be converted into fermentable sugars through enzymatic hydrolysis which then can be fermented into ethanol (Vohra et al., 2014; Vincent et al., 2015a; Hung et al., 2018). The main purpose of the pretreatment process on the TSEC is to recover more starch from both sago effluent and lignocellulosic materials in

the solid component. Figure 2.16 showed the two components of the TSEC after pretreatment process; liquid and solid components.

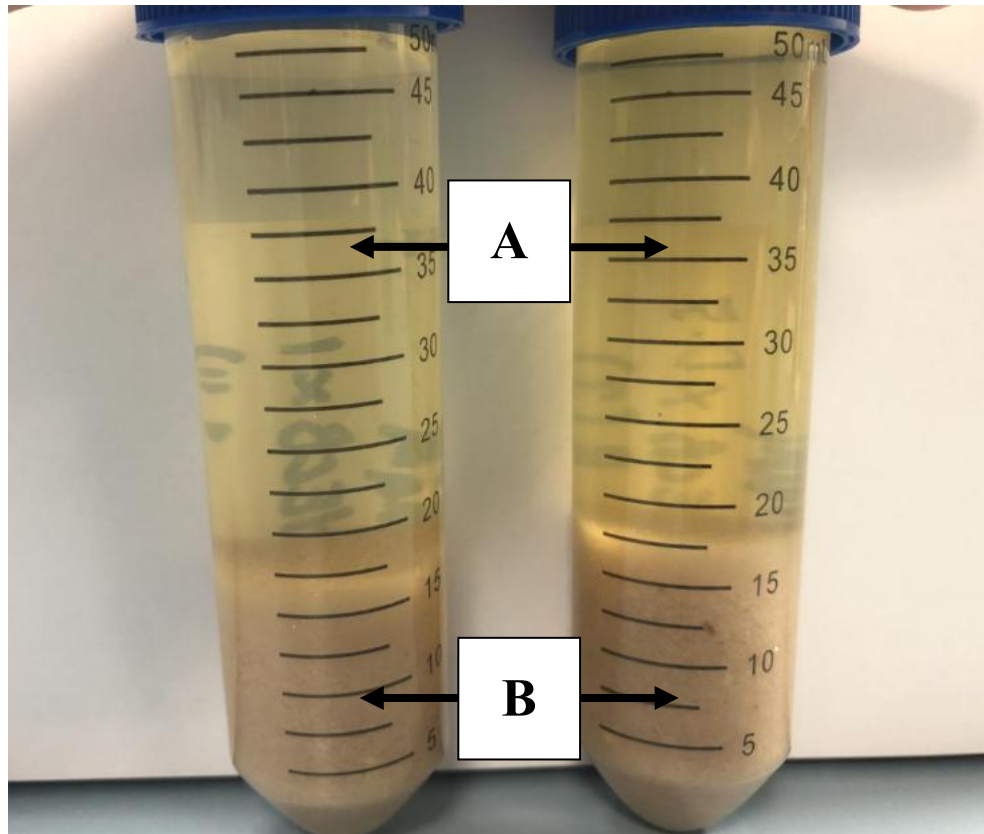


Figure 2.16: Two components of sago waste after pretreatment. A) Water component or Sago Effluent Hydrolysate; B) Solid Component or TSEC.

According to the findings by Vincent et al. in 2021, compositional analysis was done on untreated sago effluent had about 94.91% of water and 2.23% of starch in the liquid component and approximately 1.49% of starch in the solid component. As outlined

in Table 2.3, recent compositional analysis of dried sago hampas showed comparable results from previous reports. Thus, based on these reports, the TSEC as lignocellulosic biomass has a promising potential as substrate for bioethanol production as well as reducing pollution problems globally.

Table 2.3: Compositional analyses of sago hampas.

Sago hampas component (%)	Linggang et al. (2012)	Vincent et al. (2015b)	Thangavelu et al. (2016)	Hung et al. (2018)	Vincent et al. (2021)
Starch	58.0	58.0	52.0	55.4	52.0
Cellulose	23.0	23.5	16.0	23.6	27.8
Hemicellulose	9.2	8.2	9.8	9.1	5.3
Lignin	4.0	6.3	5.2	4.0	3.0

2.5 Amylase

A group of enzymes known as amylase is specifically used to break glucose bonds in starch. The groups of enzymes can be divided into four categories depending on its functionality which are endoamylases, exoamylases, debranching enzymes and transferases (El-Fallal et al., 2011). Glycosidic bond in inner part of amylose and amylopectin is commonly hydrolysed by endoamylase and the most preferred endoamylase to cut down the glycosidic bond is α -amylase (Aditiya et al., 2016). Exoamylases which is the second

group of enzymes aim the external part of amylose and amylopectin. Exoamylase such as glucoamylase are able to degrade α -1,4 and α -1,6. Debranching enzymes works on different parts of polysaccharide while transferases forms new glucosidic bonds through transfer of molecules from donor to acceptor (Aditiya et al., 2016).

2.6 Yeast

Yeasts are mainly used globally in the biotechnology industry as considerable producers compared to other groups of industrial microorganisms (Assis-Marques et al., 2015). Yeast is a group of fungi which are referred to as ascomycetous or basidiomycetous fungi that reproduce by sexual budding (Wolf et al., 1996). The morphological, physiological, and genetic characteristics (genetic reproduction) will determine the lower taxonomic subdivisions such as families, genera, species and strain (Kurtzman & Fell, 1998). Over 2500 yeast species were discovered and published by 2005. Yeasts most preferred habitat is plant and a few commensal or parasitic species found from animals. However, yeasts can also be found from the aquatic, terrestrial and aerial environment (Azhar et al., 2017). According to Phaff & Starmer in 1987, the variation of yeast species is determined by the capability of the yeasts to utilise different sources of carbon and the selectivity of nutrition as it shows substantial specialization for habitat. Depending on the

type of energy-generating process that is involved in sugar metabolism, yeasts can be determined physically into non-facultative or obligate-fermentative. Obligate-fermentative yeasts are often used in the fermentation processes due to its capability of metabolizing glucose through alcoholic fermentation (Rodrigues et al., 2006). At optimum state, the growth of yeast incline by double in 100 minutes and will slowly decline, eventually ceasing altogether under high pressure and/or environmental pressure (Byers, 1974; Ordway et al., 2020). Yeast as a model organism continues to act as an essential role in the study of fundamental biological procedures such as aging, cell cycle and stressor studies (Eardly & Timson, 2020). In emerging biotechnological area, the ability of yeast to catabolize sugars and produce bioethanol and by-product of carbon dioxide has allowed genetic modification of this microorganism to manipulate metabolic pathways to minimise catabolization of carbon to natural metabolic by-products to maximize ethanol production (Azhar et al., 2017; Eardley & Timson, 2020).

2.6.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae or also known as Baker's yeast has been related with the human species as it has been used for brewing, winemaking, alcohol-distilling, bread baking and food flavouring as well as for therapeutic purposes, consequently making *S.*

cerevisiae the most studied and understood microorganism of the yeast domain and a fundamental model system for research in the biology of eukaryotic cell (Stewart, 2014). The typical sizes of *S. cerevisiae* cells range from 7.94 μm to 10.2 μm as seen in Figure 2.17. *Saccharomyces* is referred to as sugar fungus in Latin as *Saccharomyces* is generally found in sugar-rich environments whilst, *cerevisiae* literally means “of beer” in Latin (Schneider, 2004; Gerke et al., 2006). *S. cerevisiae* species is under the genus of *Saccharomyces*, member of the family Saccharomycetaceae and subfamily of Saccharomycetoideae which is classified as unicellular, reproduce vegetatively by budding and sexually by means of ascospores (Stewart, 2014). The long history of winemaking dates back to 3.150 BC in Abydos, where the DNA of *S. cerevisiae* was discovered in wine jars from King Scorpion’s tomb (Cavalieri et al., 2003).

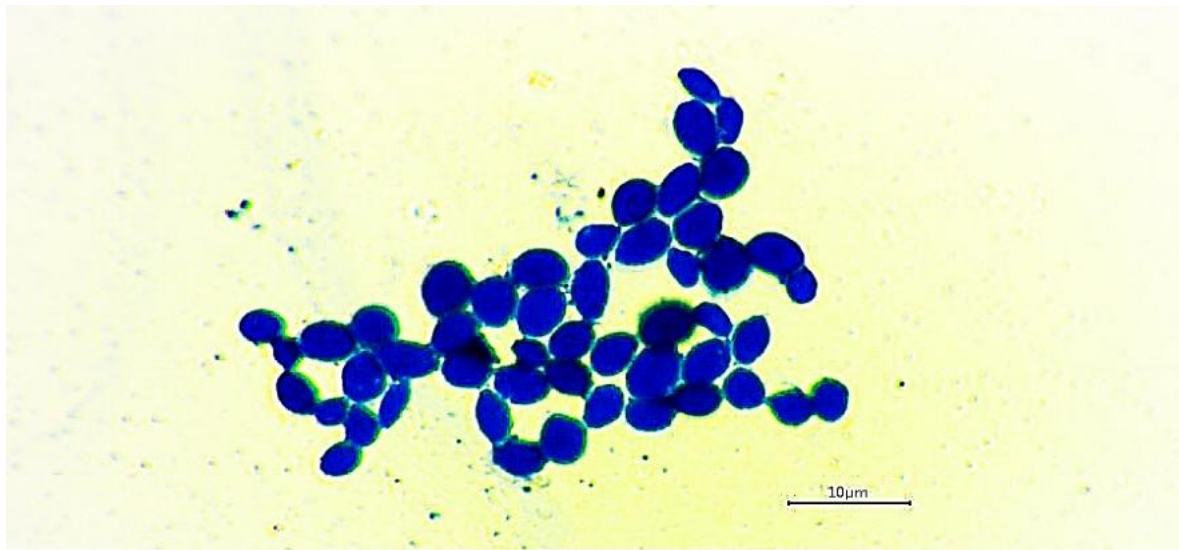


Figure 2.17: *Saccharomyces cerevisiae* captured using light microscope (OMAX DM500, Malaysia) under 1000x magnification.

Figure 2.18 showed the budding process of yeast in which the daughter cells bud off the mother cell. According to Duina et al. in 2014, *S. cerevisiae* can switch readily between two mating types; haploid and diploid, in nature. The unique characteristic of cell division distinguishes *S. cerevisiae* from another powerful organism, *Schizosaccharomyces pombe*. The life cycle of *S. cerevisiae* is mostly dependent on growth and survival strategies. In a known natural niche, haploids undergo asexual reproduction of simple mitosis whilst, under stress condition, diploids will go through meiosis and sporulation to form four haploid spores (Duina et al., 2014; Parapouli et al., 2020).

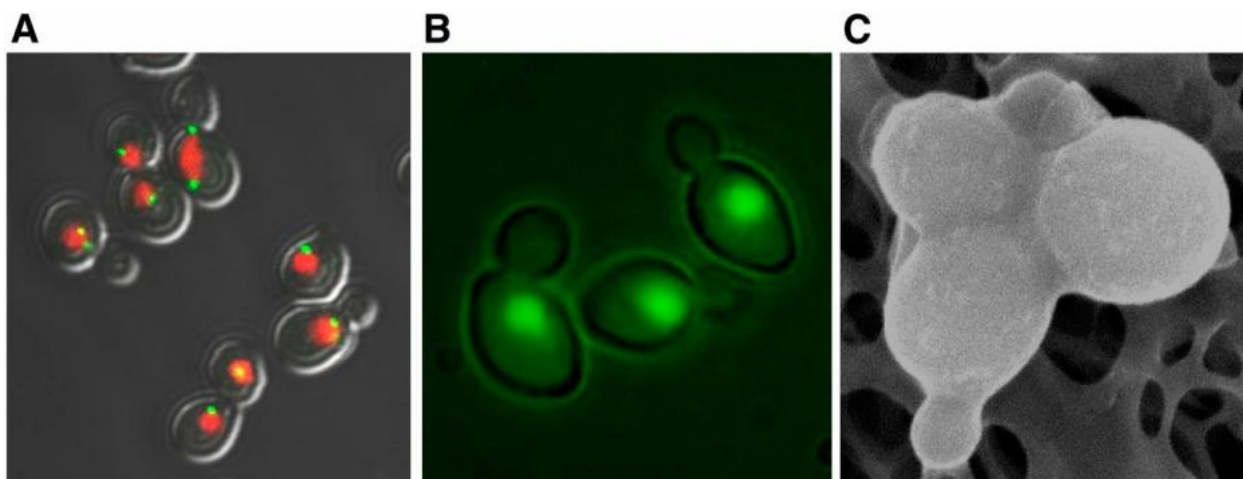


Figure 2.18: A) Haploid yeast cells under fluorescence microscope; B) Diploid yeast cells under epifluorescence microscope; C) Dividing yeast cells under electron microscope (Duina et al., 2014).

S. cerevisiae is robust yeast widely used in the ethanol production industry due its tolerance towards a broad range of pH and stressful condition hence, making the process less contaminated (Lin et al., 2012). According to Andrietta et al. (2007), *S. cerevisiae* also has a high fermentation efficiency, rapid growth, efficient use of sugar, ability to produce and consume ethanol, tolerance to high concentrations of ethanol and low levels of oxygen, osmotolerance, thermotolerance and cell activity in acidic conditions, which are important to its industrial utility. *Saccharomyces* yeasts have a variety of responses to allow survival in deleterious circumstances, including filamentation, invasive production, flocculation and creation of biofilms that are undesirable to the industry (Fildago et al., 2006).

According to a study done by Wang et al. (2012), eight new lineages of wild *S. cerevisiae* named as CHN I to CHN VIII were isolated from environments near and far from human activity. Nevertheless, due to the presence of these wild strains of *S. cerevisiae*, contaminations occur during industrial processes as Baker's yeast and other strains of the same genus are unable to compete with it (Azhar et al., 2017). It is often a technological challenge to achieve maximum ethanol production however, as stressful environments like microorganism contamination, osmotic and cellular pressure, temperature and raw material impurities affect yeast productivity (Azhar et al., 2017; Eardley and Timson, 2020). Notably, the growth rate for yeasts and its metabolism incline as the temperature rises until the optimum value. Despite its tolerance to a wide range of pH, increase in temperature (35-45 °C) and high ethanol concentration (>20%) are common obstacles for yeast and *S. cerevisiae* during sugar fermentation. *Saccharomyces* specific characteristic appears to be its tolerance to high ethanol concentration about at 8-12% ethanol (v/v) and varies according to different strains of *S. cerevisiae* (Azhar et al., 2017). Fluctuating condition of ethanol during fermentation may promote sensitivity of *S. cerevisiae* to alcohol-rich media/broth or anaerobic condition which leads to rise of temperature (>30 °C or <10 °C) which would induce the biosynthesis of stress response factor and eventually inhibit further yield (Stewart, 2014; Azhar et al., 2017).

The main property of *Saccharomyces* species is its ability to ferment hexose sugars (glucose, fructose and mannose) in alcoholic fermentation (Table 2.4). Other types of fermentable sugar include sucrose, maltose and galactose whereas only specialized species *Saccharomyces* can ferment dextrins and starch (Azhar et al., 2017). On the contrary, pentose sugars are considered non fermentable for *S. cerevisiae*. Hence, only yeast from genera *Candida*, *Pichia*, *Pachysolen* and *Schizosaccharomyces* are competent to metabolize pentoses to ethanol (Stewart, 2014; Azhar et al., 2017).

Table 2.4: Key properties for the identification of *S. cerevisiae* (Stewart, 2014).

Property	<i>S. cerevisiae</i>
Fermentation	
Sucrose	+
Raffinose	+
Trehalose	-
Assimilation	
Sucrose	+
Maltose	+
Raffinose	+
D-Ribose	-
Ethanol	+
D-Mannitol	-

Ethanol fermentation is synonymous to *S. cerevisiae*, the main biocatalyst in fermentation of sugar in the industry of ethanol production. It is noteworthy, although *S. cerevisiae* is a valuable species for all aspects of basic research, it is difficult to satisfy the demands among the parameters in the biorefineries industry. Genetically modified or metabolically engineered *S. cerevisiae* improves strains to synthesize alcohols such as propanol and butanol besides bioethanol (Nandy & Srivastava, 2018). Kim et al. (2013) have been meticulously discussed and reviewed the genetic manipulation and mechanisms of *S. cerevisiae* and the strain improvement has been implemented by companies that developed commercial production of isobutanol such as Butalco, Butamax and Gevoare. Optimized strain has been extensively discussed and includes improved rate of ethanol yield, ethanol and stress tolerance, utilisation of xylose, acetic acid resistance and effective adaptation for large scale fermentations in second generation bioethanol industry (Nandy & Srivastava, 2018; Parapouli et al., 2020). Despite the efficiency of various *S. cerevisiae* strains used throughout the process, there are more to be discussed and considered either genetically engineering existing strains or risk evaluations of environmental exploitation of natural reservoirs (Schuller et al., 2005; Parapouli et al., 2020).

2.7 Fed-Batch Fermentation

Fermentation is an ancient technology used in the field of biotechnology and often used in the industrial sector to produce biofuels, biopharmaceuticals, chemical building blocks, food and feed supplements (Ochoa, 2019; Yang & Sha, 2019). Main objective of fermentation research is to develop economical production of bioproducts with desired efficiency and yield (Lee et al., 1999; Yang & Sha, 2019). According to Chin & H'ng (2013) and Ochoa (2019), fermentation can be carried through three different procedures depending on the microbial kinetic properties and type of feedstock; batch, fed-batch and continuous which are differentiated by the reactor in industrial fermentation (Figure 2.19).

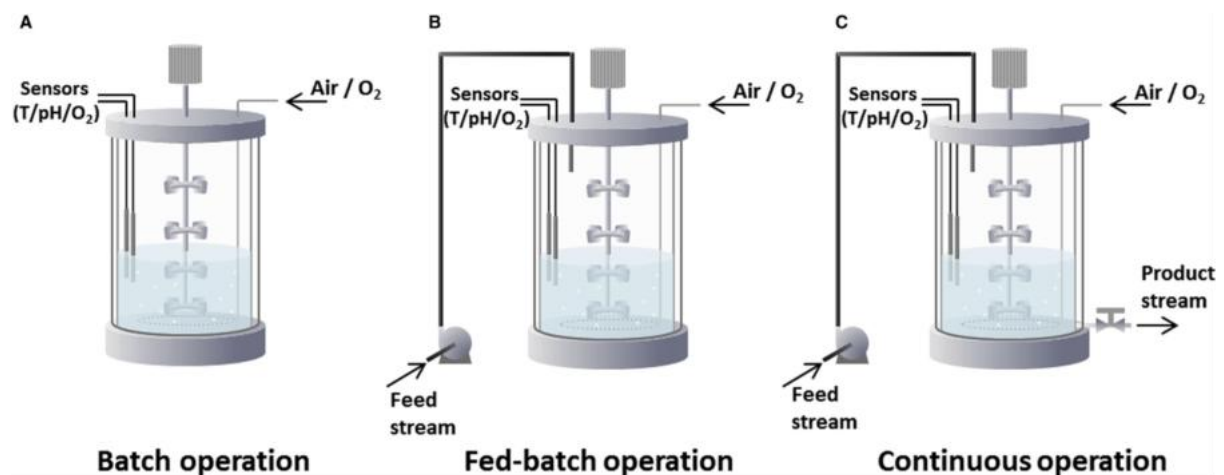
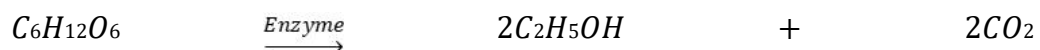


Figure 2.19: Graphical comparison of three different modes of fermentation process: (A) batch (B) fed-batch and (C) continuous (Ochoa, 2019).

The chemical reaction (Yang & Sha, 2019) as shown in Figure 2.20:



(Glucose)

(Bioethanol)

CarbonDioxide

Fed-batch fermentation is an adaptation technique of batch fermentation and is commonly used in bioprocess technology (Yang & Sha, 2019). According to the research done by Lee et al. (1999) and discussed thoroughly by Yang & Sha (2019), required microorganisms and nutrient sources are added in fermentation broth or medium under batch process phase. Subsequently, one or more nutrients is added in increments to the broth or medium throughout the fed-batch process and the products remain in the broth or medium until the end of the process. Addition of substrate and/or nutrient also referred to as feeding is determined by feeding policy by means of designated time profile to maintain suitable amount of substrate and microbial growth in the remaining duration of fermentation. Byproducts of fed-batch fermentation are yielded at the end of the process which results in substantial accumulation of biomass produced during the exponential growth phase due to the addition of substrate (Ochoa, 2019; Yang & Sha, 2019).

It was reported by Chang et al. (2012), fed-batch fermentation process is ideally used for ethanol production of lignocellulosic raw materials such as corn stover and recycled paper-derived materials. The fed-batch system maintains the dynamic environment during fermentation process and helps to prevent inhibition catabolite repression (Cheng et al., 2009; Hadiyanto et al., 2013). Besides, in comparison with the

batch system, the fed-batch system is more advantageous in controlling microbial rate, higher ethanol yield with shorter fermentation time, higher saccharification and dissolved oxygen rate (Chang et al., 2012; Moslamy, 2019).

2.8 Simultaneous Saccharification and Fermentation (SSF)

The SSF is a process of both saccharification and fermentation are performed at the same time in single vessel (Vohra et al., 2014; Srivastava et al., 2020). By combining steps, the process reduces the need of enzymes, fermentation period and also overall production cost. Moreover, the SSF reduces the risk of microbial contamination as well as end-product inhibitors (Guo et al., 2021). The main advantage of the SSF process is its ability to convert sugar to ethanol rapidly thereby enhances ethanol yields. According to Liu et al. (2014), the SSF process yielded more ethanol which about 5.1% compared to the Separate Hydrolysis and Fermentation (SHF) process. However, the temperature varies among process as the optimum temperature for the entire process is lower than the saccharification temperature thus, it may reduce the efficiency of enzymatic hydrolysis which will results for more enzymes (Guo et al., 2021). To date, the SSF is the most convenient method for bioethanol production at both laboratory and industrial platforms (Srivastava et al., 2020).

2.9 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is based on the theory of Liquid Chromatography (LC) and or the instrument of Gas Chromatography (GS) (Lindsay, 1992). HPLC involves a column chromatography that is commonly used in analysis, biochemistry

and manufacturing to isolate, identify and measure active compounds. HPLC mainly uses column that hold stationary phase material, mobile phase moving pumps through the column and detectors that indicate the molecules retention time (Malviya et al., 2010) (Figure 2.21). The basic principle of column chromatography is that molecules can dissolve in liquids as well as with solid surface and it purify compounds based on their polarity. The molecules separation by chromatography is largely depends on several physical properties of molecules in the sample such as the solubility of organic solvents, the size and net positive or negative charge (Bird, 1989). Thus, the molecules in the sample will have different affinities and interactions with the stationary support (Kupiec, 2004).

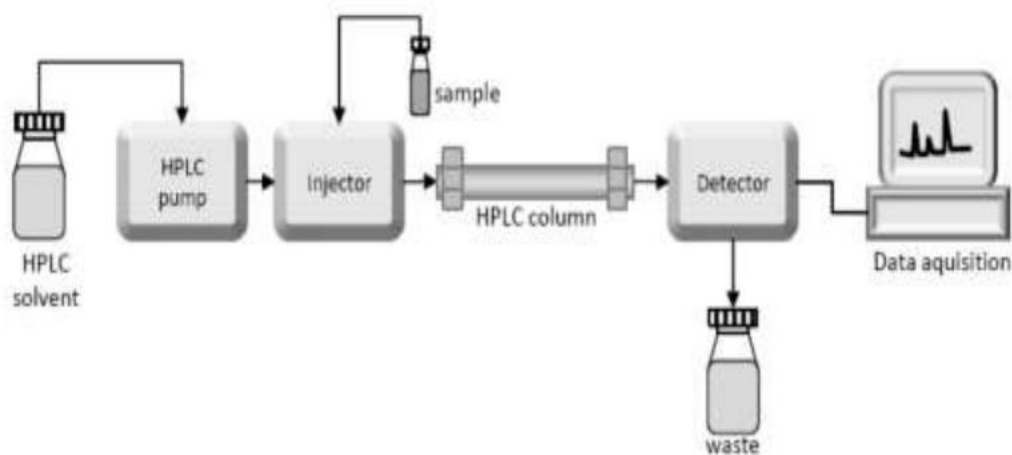


Figure 2.21: The flow diagram of High Performance Liquid Chromatography (HPLC)

(Czaplicki, 2013).

The HPLC column is usually made of silica that is greatly compact in nature. The silica particles can perform its function because of the long carbon chains as the chains are non-polar. Hence, the longer the chain, the less polar the column will be (Malviya et al., 2010). As the sample crosses the column, the analysis that has the strongest interface with the stationary phase exits the column the slowest, meaning it shows the longest retention time. On the contrary, the sample that shows little interaction with the bending material and is characterized to have shorter retention time. The compounds separation can be accomplished through isocratic elution, where the mobile phase composition remains constant or through the gradient elution, where the mobile phase composition changes throughout the separation to the state in favour of analytical separation from the stationary phase (Petrova & Sauer, 2017). Once exiting the column, the mobile phase permits through a detection module such as fluorimeter or a UV absorbing detector. Appropriate selection of detector and observing wavelengths is important for optimizing HPLC detection sensitivity (Petrova & Sauer, 2017).

2.10 Phenol-sulfuric Acid (PSA) Assay

Phenol-sulfuric acid (PSA) assay created by Dubois et al. (1951) is a process for carbohydrate analysis. PSA assay is capable of detecting all classes of carbohydrates

including monosaccharides, disaccharides, oligosaccharides and polysaccharides which is then distinguished according to its absorptivity level (Nielsen, 2017). The absorptivity level varies between different carbohydrates and hence, enabling the carbohydrates to be distinguished. This method can be used to measure reducing and non-reducing sugar as it is the most reliable method to analyse total carbohydrate in a sample because of its sensitivity and simplicity (Masuko et al., 2005).

In this process, the intensity of colour is depending on the different classes of sugar such as pentoses and hexoses. According to Figure 2.22, coloured complexes are formed upon the addition of phenol as the carbohydrates in the solution are heated and dehydrated with concentrated sulphuric acid and generate furan derivatives (Jain et al., 2017; Arenja et al, 2018). The reaction continues as the formation of furan derivatives such as hydroxymethylfurfural produces a yellow-gold compound which can be detected using a spectrophotometer and has the absorption maximum at 490 nm under UV spectrophotometer (Jain et al, 2017; Nielsen, 2017). In addition, according to Dubois et al. (1951), the reagents used in the standard procedure of the PSA are low cost and readily available. Hence, this method is very helpful for environmental studies as it can reduce the time consumed.

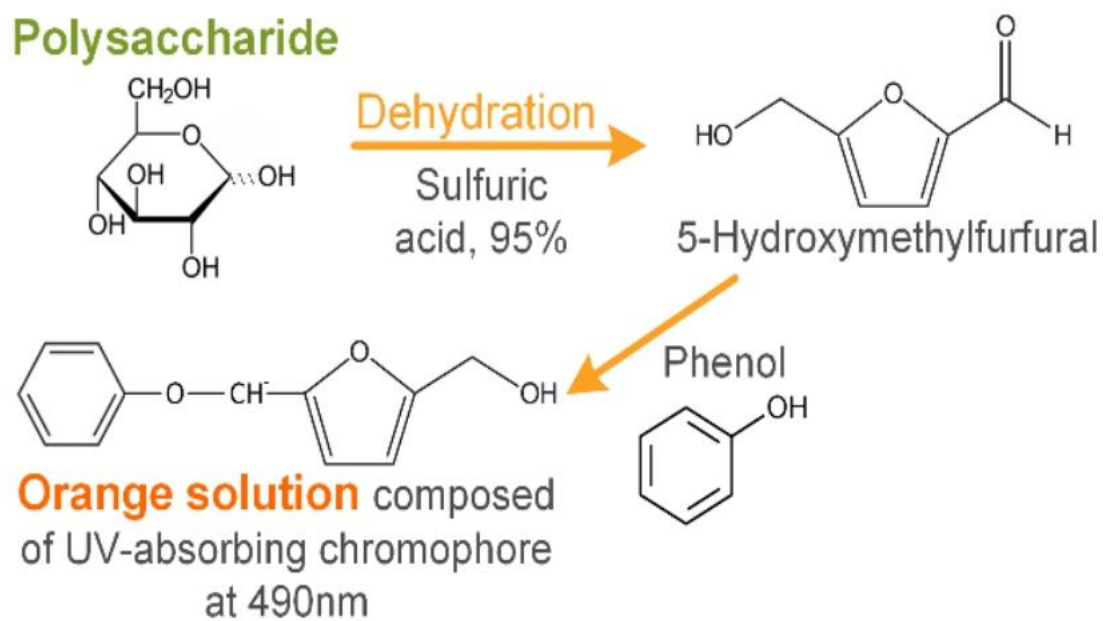


Figure 2.22: Schematic reactions of Phenol-sulfuric Acid (PSA) assay for the total carbohydrates analysis (Viel et al., 2018).

CHAPTER 3

METHODOLOGY

3.1 Experimental Setup

Figure 3.1 shows an overview of experimental procedures in this study.

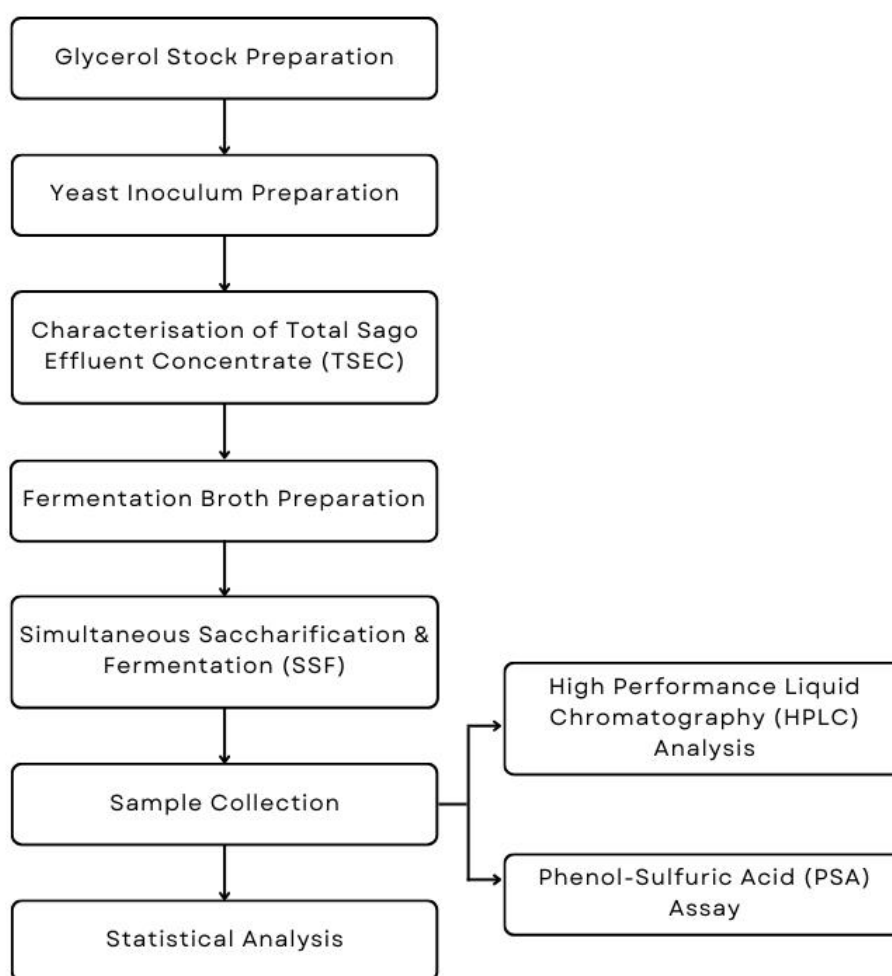


Figure 3.1: Flowchart of experimental design.

3.2 Glycerol Stock Preparation

Saccharomyces cerevisiae (ATCC 24859) was revived from stock culture stored in Microbiology Laboratory of Faculty of Resource Science and Technology, UNIMAS in Yeast Malt Broth (YMB) (Himedia Laboratory, Mumbai, India) at ambient temperature (20 to 25 °C) on rotary shaker (Daihan Scientific, South Korea) at 150 rpm for 24 hours. Theoretically *S. cerevisiae* appeared as cloudy in YMB after 24 hours thus, was subcultured on Rose Bengal Chloramphenicol Agar (RBCD) (Himedia Laboratory, Mumbai, India) and left to grow for 48 hours at room temperature. Prior to further analyses, the colonies were taken for microscopic observation and also were subcultured in YMB overnight for glycerol stock preparation. For long-term storage, the overnight stock culture was aliquoted in YMB supplemented with 20% (v/v) glycerol (RandM Chemicals, United Kingdom) and stored in ultra-low-temperature freezer (Sanyo-MDF-U500VX-PE Wolf Laboratories Ltd, Pocklington, United Kingdom) at -20 °C (Vincent, 2010).

3.3 Yeast Inoculum Preparation

Yeast inoculum was prepared by growing the glycerol stocks in YM broth overnight at ambient temperature on orbital shaker at 150 rpm. The cells were harvested through centrifugation (BK-1032J Low Speed Centrifuge, Biobase, China) (Figure 3.2) in

separated from the sago effluent through a centrifugation process in four 50 mL centrifuge tubes for 10 minutes at 4000 rpm. Subsequently, suspended solids and supernatants were collected and transferred into two sterilised bottles for long-term storage (Figure 3.3).

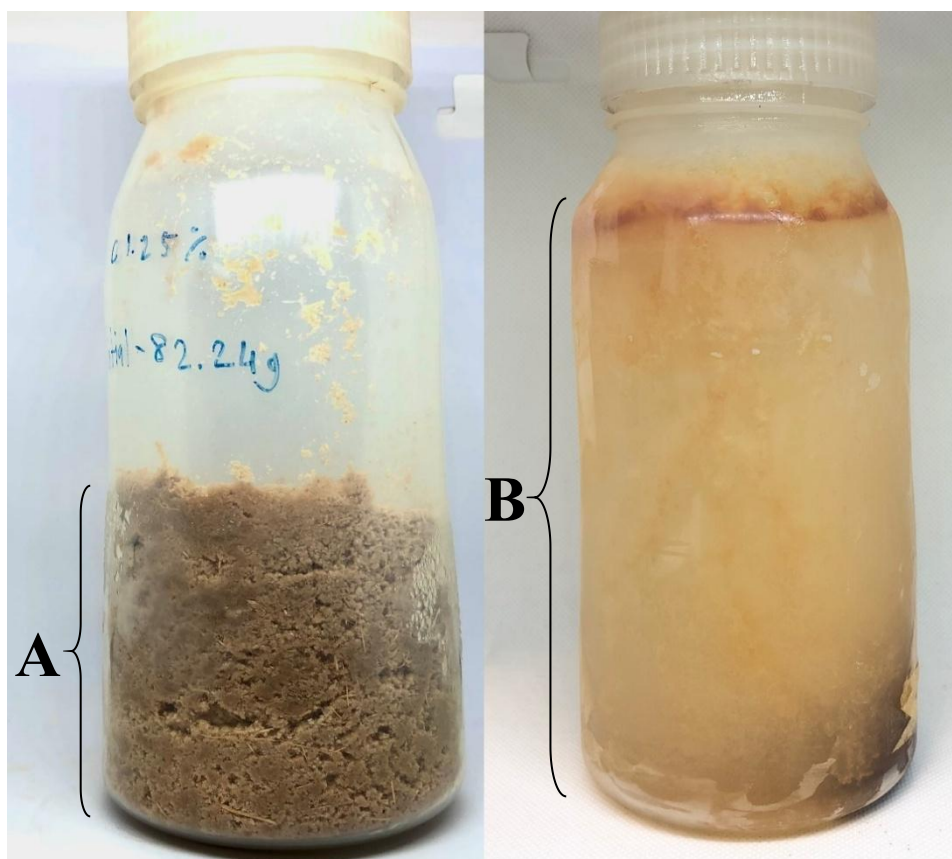


Figure 3.3: A) Solid component or Total Sago Effluent Concentrate (TSEC); B) Liquid component or sago hydrolysate.

3.4 Characterisation of Sago Hampas

3.4.1 Determination of Moisture Content

The moisture content of sago hampas was determined by using equation 3.1 after oven drying (Shel Lab, USA) at 105 °C for 3 days.

$$\text{Moisture content \%} = \frac{(\text{weight of wet hampas} - \text{weight of dry hampas})}{\text{weight of dry hampas}} \times 100 \quad \text{Equation 3.1}$$

The total sago effluent concentrate (TSEC) was destarched and oven-dried before it was ground to fine powder (Figure 3.4). The powdered sample was subjected for starch and fibre analysis using methods Phenol-sulfuric Acid (PSA) assay, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF) and Klason Lignin Determination. Ash content was determined using a furnace incineration method where the sample was dry ashed at 550 °C for 6 hours by ashing muffle furnace (KC, 40/13, ThermoConcept, Germany) until ash was obtained.

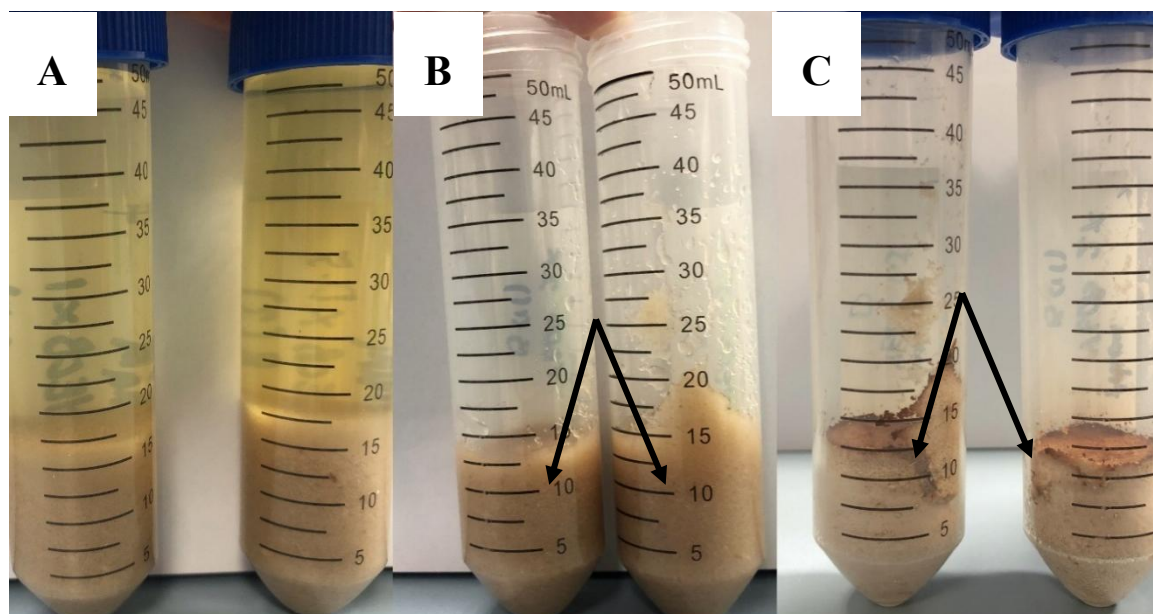


Figure 3.4: A) TSEC was separated to two components after centrifugation; B) Wet solid component of TSEC after sago hydrolysate was removed; C) Solid component of TSEC after oven-dried for 3 days.

3.5 Fermentation Broth Preparation

Fermentation broths were prepared according to Table 3.1 using modification methods from Vincent et al. (2011).

Table 3.1: The ratio of fermentation broth preparation.

	Control (mL)	25 mM (mL)	50 mM (mL)	100 mM (mL)
2M Citrate Buffer	-	1.25	2.5	5.0
Total Sago Effluent Concentrate (TSEC)	20.0	20.0	20.0	20.0
Effluent	80.0	78.8	77.5	75.0
Total	100.0	100.0	100.0	100.0

Fermentation broths were prepared in batch culture of 100 mL with the pH maintained between 4.8 and 5.4 containing the mixture of TSEC, effluent and 2 M of citrate buffer except for control fermentation broth. The fermentation broths were autoclaved (25X Electric Pressure Steam Sterilizer, All American, USA) for 15 minutes at 121 °C.

3.6 Simultaneous Saccharification and Fermentation (SSF)

The sterilised fermentation broths were added with 75 µl of α -amylase (Sunson Industry Group, China) and gluco-amylase (Sunson Industry Group, China) subsequently, incubated for an hour at ambient temperature (20 to 25 °C). Harvested cells of *S. cerevisiae* were also added into the fermentation broths. Fermentation processes were conducted in triplicates (n=3) as presented on Figure 3.5 for 5 days at ambient temperature on a magnetic stirrer (48-I, Biobase Industry, China) with constant agitation of 150 rpm. Prior to the fed-batch fermentation process, 5 g of TSEC and 20 µl of α -amylase and gluco-amylase were added into the fermentation broths aseptically at every 24, 48 and 72 h.

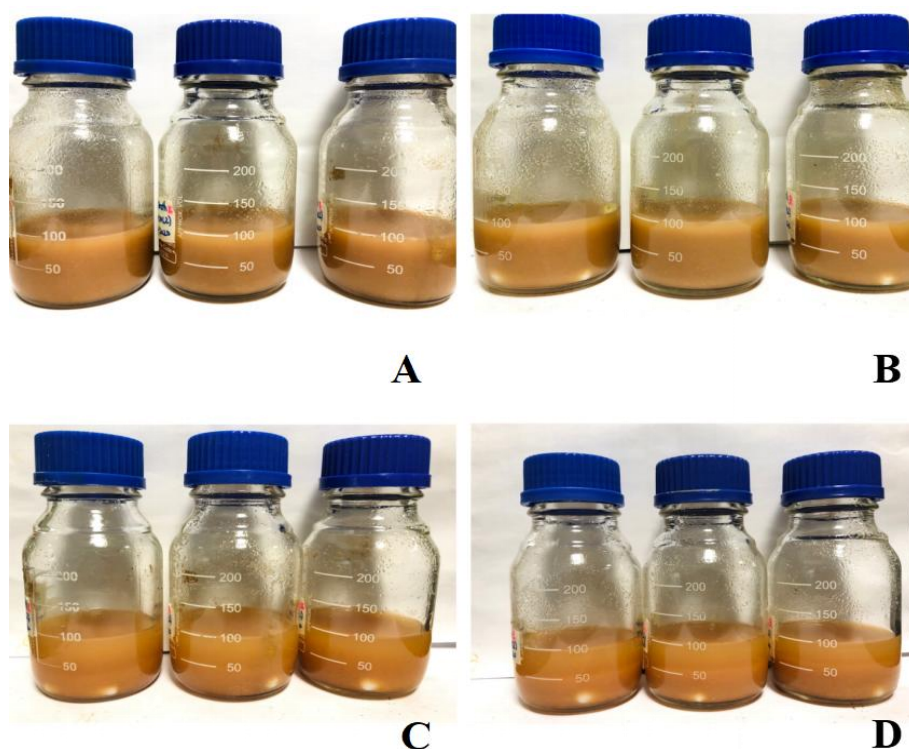


Figure 3.5: Fermentation broths at 0 h containing TSEC in (A) Non-buffered broth; (B) 25 mM buffered broth; (C) 50 mM buffered broth; (D) 100 mM buffered broth.

3.7 Sample Collection

At 0, 12, 24, 36, 48, 60, 72, 84, 96 and 120 h, 2.0 mL of samples were pipetted out aseptically and transferred into 2.0 mL centrifuge tubes. The samples for PSA assay and HPLC analysis were initially centrifuged at 13500 rpm for 10 minutes using microcentrifuge (*WiseSpin* CF-10, Daihan Scientific, South Korea) and the supernatants were filtered through 0.45 μm nylon membrane filter (Whatman, NJ, USA) to eliminate solid matter. The filtered samples were stored at $-20\text{ }^{\circ}\text{C}$ until further analyses were done.

3.8 High Performance Liquid Chromatography (HPLC) Analysis

The detection and quantification of ethanol, residual glucose, lactic acid and acetic acid from fermentation samples were analyzed according to standard protocol using HPLC (Waters 2695 Separations Module, Alliance HPLC System, USA). The HPLC system included a column heater, refractive index detection and a computer controller. The fermentation constituents were separated and analyzed on a Bio-Rad Aminex HPX-8711 column (150 × 7.8 mm; Bio-Rad, USA) with 5 mM sulphuric acid (H₂SO₄) as the mobile phase, at a flow rate of 0.8 mL/min and a temperature of 65 °C (Hung et al., 2018; Vincent et al., 2015b). The concentration of components was determined based on a standard concentration curve according to retention time (Table 3.2).

Table 3.2: The retention times of components via HPLC analysis.

Components	Retention time (min)
Cellobiose	3.141
Glucose	3.820
Xylose	4.048
Lactic Acid	5.144
Acetic Acid	6.017
Ethanol	8.804

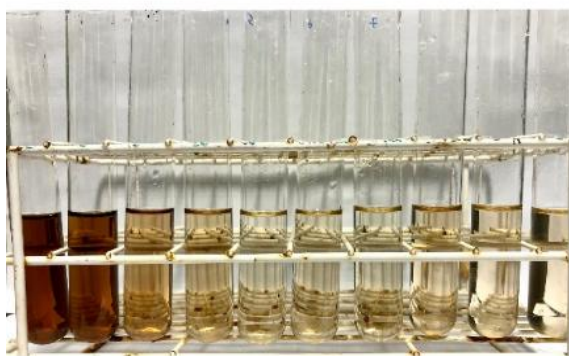
3.9 Phenol-sulfuric Acid (PSA) Assay

The supernatants were diluted to 1:5 ratios and mixed well using vortex (VX-200, Labnet International Inc., USA). Subsequently, 0.2 mL of the dilutions was dispensed into new test tubes. Next, 0.2 mL of 5% phenol (Merck KGaA, Germany) was added into each

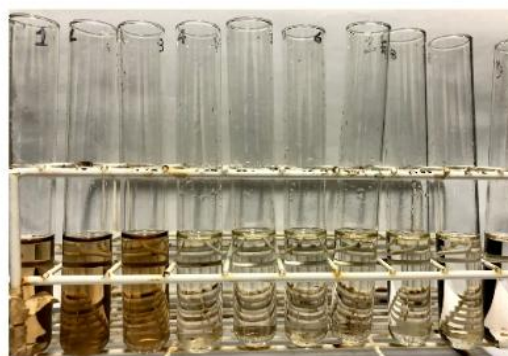
tube and mixed well by vortexing. 1 mL of 98% sulphuric acid (R&M, United Kingdom) was added and mixed well. Lastly, 5.6 mL of distilled water was added into each tube to the final volume of 7 mL and followed by final mixing by vortex. The PSA assay were conducted in triplicate (n=3) as presented in Figure 3.6. The absorbance of the solutions was measured at 490 nm using a spectrophotometer (SP-880, Metertech Inc., Taiwan). The equivalent sugar concentration (g/L) was determined according to an initial glucose standard curve tabulated as presented in the Appendices prior to the assay.

Figure 3.6: Colour development of Phenol-sulfuric Acid Assay using TSEC as substrate in

A) Non-buffered Broth; B) 25 mM Buffered Broth C) 50 mM Buffered Broth;



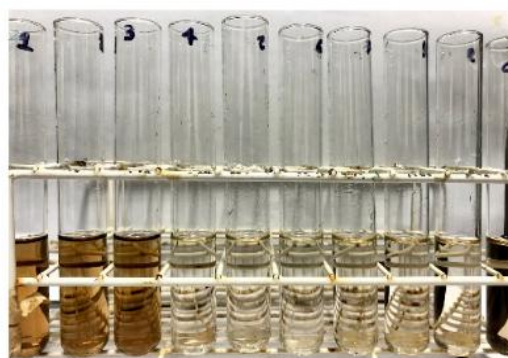
A



B



C



D

C) 100 mM Buffered Broth.

3.10 Statistical Analysis

Tukey's post hoc tests were used in statistical analysis with SPSS Statistics Software version 21 to analyze the statistically significant differences in theoretical ethanol yields from different buffer strength.

CHAPTER 4

RESULTS

4.1 Characterisation of the Total Sago Effluent Concentrate (TSEC)

In this study, composition analyses were done prior to the Simultaneous Saccharification and Fermentation (SSF) process and the data obtained are presented in Table 4.1 and 4.2. Table 4.1 shows the compositions of Total Sago Effluent Concentrate (TSEC) which comprises $88.11 \pm 2.09\%$ sago hydrolysate and $1.89 \pm 0.14\%$ dry sago hampas. In summary, Table 4.2 shows the main components of dried sago hampas which contained $65.80 \pm 4.25\%$ starch, $5.95 \pm 1.53\%$ cellulose, $18.20 \pm 1.60\%$ hemicellulose, $5.25 \pm 0.70\%$ lignin and $0.58 \pm 0.01\%$ ash in dry basis (w/w). The percentage of starch content in TSEC is high compared to previous research done by Thangavelu et al. (2014), Hung et al. (2018) and Vincent et al. (2021) which showed approximately 58.0%, 55.4% and 52.0% of starch respectively.

Table 4.1: Component analysis of Total Sago Effluent Concentrate (TSEC) comprising of sago hydrolysate and dried sago hampas, n=3.

Composition		Amount (%)	
Total Sago Effluent Concentrate (TSEC)	Sago effluent hydrolysate (Water Component)	Water	88.11 ± 2.09
		Free starch	1.31 ± 0.05
		Starch	3.56 ± 0.80
		Cellulose	0.32 ± 0.08
	Dried Sago Hampas (Solid Component)	Hemicellulose	1.07 ± 0.08
		Lignin	0.28 ± 0.04
		Ash	0.03 ± 0.02
		Others	5.33 ± 0.01

Table 4.2: Component analysis of dried sago hampas. The percentage was based on dry weight, n=3.

Compositions	% w/w
Starch	65.80 ± 4.25
Cellulose	5.95 ± 1.53
Hemicellulose	18.20 ± 1.60
Lignin	5.25 ± 0.70
Ash	0.58 ± 0.40
Other	4.22 ± 0.01

4.2 Phenol-sulfuric Total Carbohydrate Assay

Phenol-sulfuric acid assay was done to determine the total carbohydrate concentration in this study. The results are depicted in Figure 4.1 whereby the initial carbohydrate for the non-buffered broth, 25 mM, 50mM and 100 mM buffered broth are 39.83, 34.08, 36.91 and 34.18 g/L respectively. The total carbohydrates in all fermentation

broths decreased steeply in the first 48 h. Prior to fed-batch fermentation, 5 g of TSEC and 20 μ l amylolytic enzymes (α -amylase & glucoamylase) were added every 24, 48 and 72 h. Thus, at 60 and 84 h, there are slightly raised of total carbohydrates concentration which later gradually declined at 96 h. However, not all sugars are converted into ethanol based on the result obtained from the experiment which residual carbohydrates were still detectable in all fermentation broths at 120 h.

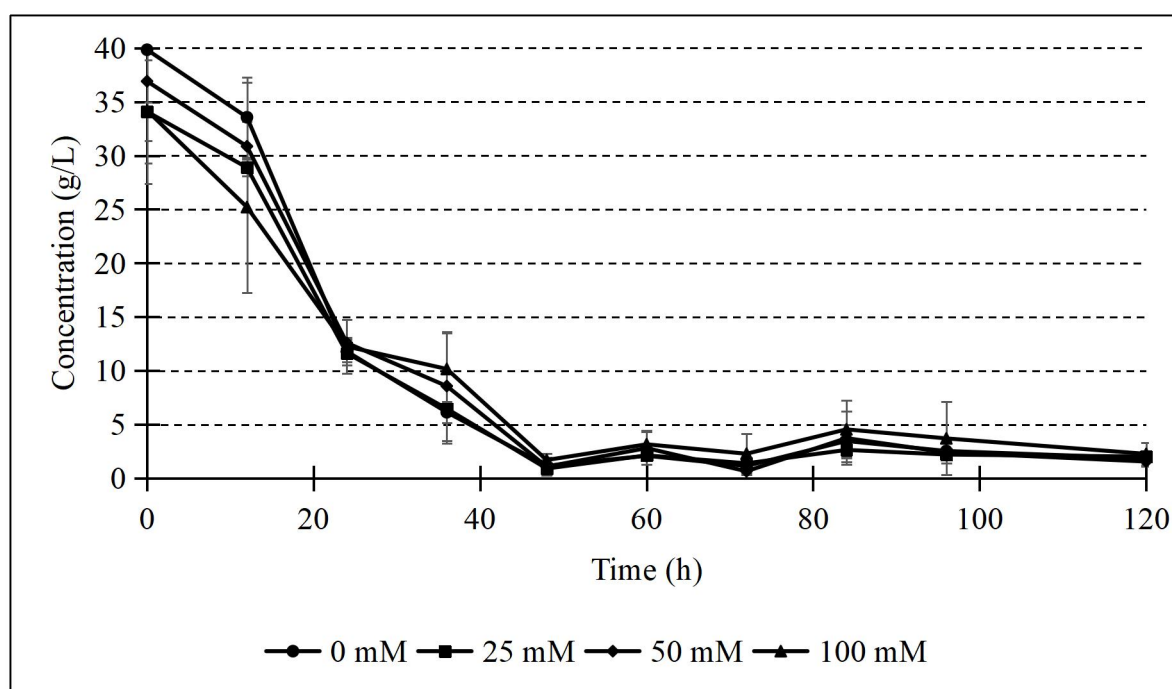


Figure 4.1: Time course of total carbohydrate content in broths containing TSEC with different strengths of citrate buffer during fed-batch bioethanol fermentation, as determined by the Phenol-sulfuric acid assay during bioethanol fermentation. [Note: The data points represent the average of three independent experiments (n=3).]

4.3 Simultaneous Saccharification and Fermentation (SSF)

The concentration of ethanol, glucose, lactic acid and acetic acid were analysed using High Performance Liquid Chromatography (HPLC). The concentration of ethanol produced during the fermentation using different media is shown in Figure 4.2. The production of ethanol is a key indicator in evaluating the ability of *Saccharomyces cerevisiae* in yielding ethanol in different buffer strengths which included 25, 50 and 100 mM. Although the ethanol concentration produced by every fermentation varied, the highest concentration was all achieved at 96 h with values ranging from 20.61 (in 100 mM buffered broth) to 23.45 g/L (non-buffered broth). According to the results, ethanol production started initially at 12 h in all fermentation broth. The ethanol concentration in all fermentation continued to increase until 96 h and declined at the end of the fermentation period.

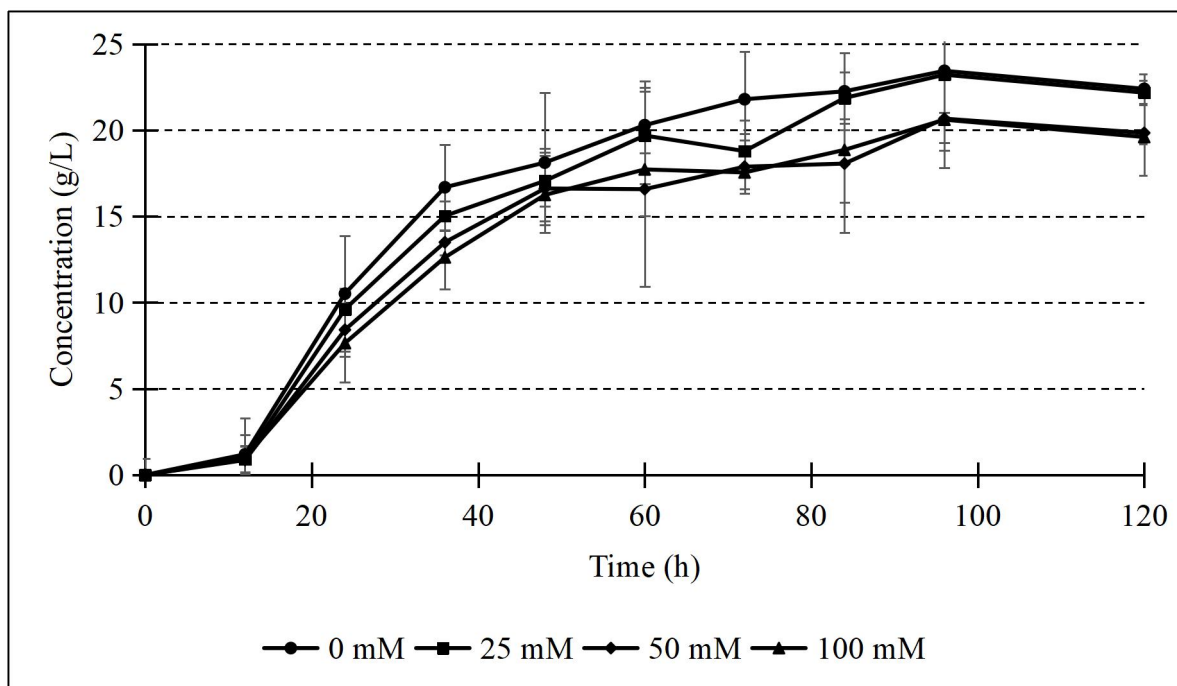


Figure 4.2: Time course of ethanol concentration in broths containing TSEC with different strengths of citrate buffer during fed-batch bioethanol fermentation, as determined by HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

Figure 4.3 depicts the glucose concentration profiles. The initial concentration of glucose was 37.30, 35.431, 31.31 and 33.05 g/L for the non-buffered broth, 25, 50 and 100 mM. In the non-buffered broth, the glucose concentration declined from 37.30 to 4.80 g/L within 36 h. After 48 h, no glucose was detected and glucose concentration continues to incline slightly at 60 h. The same pattern followed at 72 h and 84 h before glucose concentration completely undetectable until the end of the fermentation period. In contrary to buffered broths, different strengths of buffer showed different patterns in glucose concentration profiles. For 25 mM buffered broth, the glucose concentration declined from 35.43 to 5.08 g/L at 36 h and no glucose detected from 48 h to 72 h. The glucose concentration

continued to increase slightly at 84 h and further declined until 120 h. The glucose concentration profile for 50 mM buffered broth, the glucose decreased from 31.31 to 0.91g/L at 60 h and at 72 h, the glucose was completely consumed. The glucose concentration had increased at 84 h and until 120 h, no glucose was detected. Lastly, as for glucose in 100 mM, the concentration decreased from 33.05 to 1.64 g/L at 48 h. The pattern of glucose profiles at 60 h until 120 h was similar with the non-buffered broth however, the glucose was not completely consumed in 100 mM buffered broth. The glucose concentration was still detectable until the end of fermentation hence, indicated the glucose consumption was not 100% (120 h).

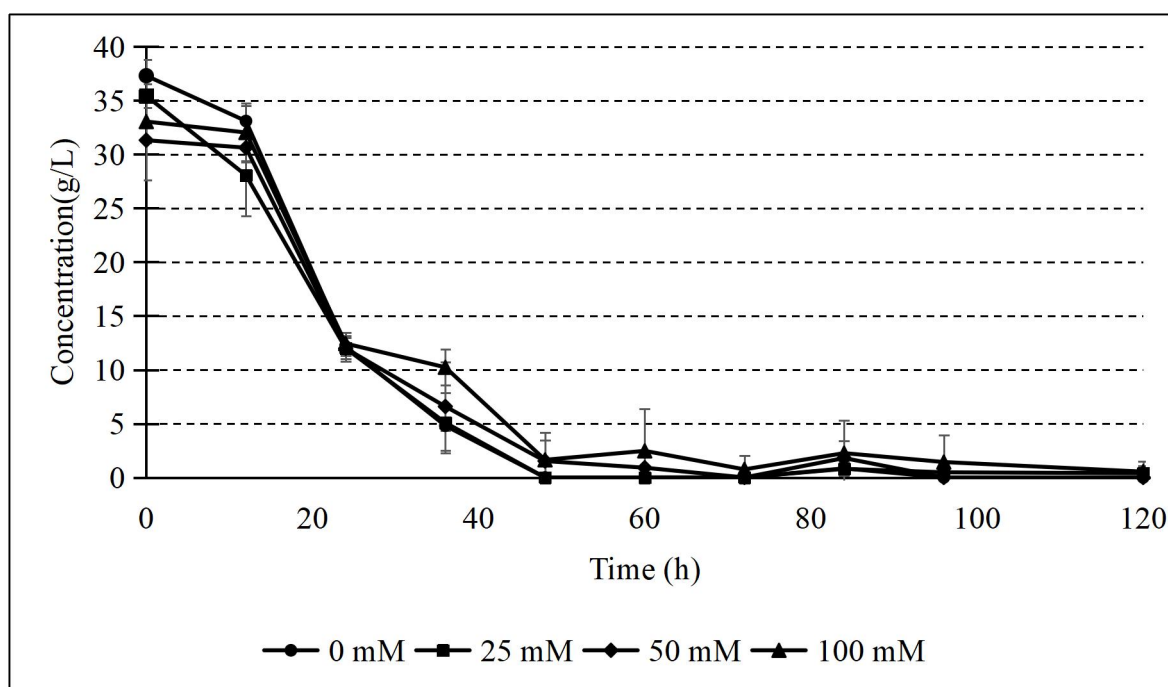


Figure 4.3: Time course of glucose concentration in broths containing TSEC with different strengths of citrate buffer during fed-batch bioethanol fermentation, as determined by HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

The concentrations of by-products from fermentation were also quantified by HPLC. The production of lactic acid gradually surged after 24 h and peaked at 120 h in all fermentation broth. However, in 100 mM buffered broth, lactic acid production was higher compared to other fermentation broths (Figure 4.4). At 120 h, the highest lactic acid concentration in the non-buffered broth, 25 and 50 mM were 4.18, 4.21 and 4.30 g/L, respectively. Whilst in 100 mM buffered broth, the highest concentration of lactic acid was 6.03 g/L. In this current study, the lactic acid concentrations were in the range 0.19 to 6.03 g/L and according to Hung et al. in 2018, the inhibition of ethanol production only occur once the concentration of lactic acid is above 30 g/L or 3.00% (w/v). Thus, the yeast metabolism and ethanol yield in all fermentation were not affected by lactic acid production.

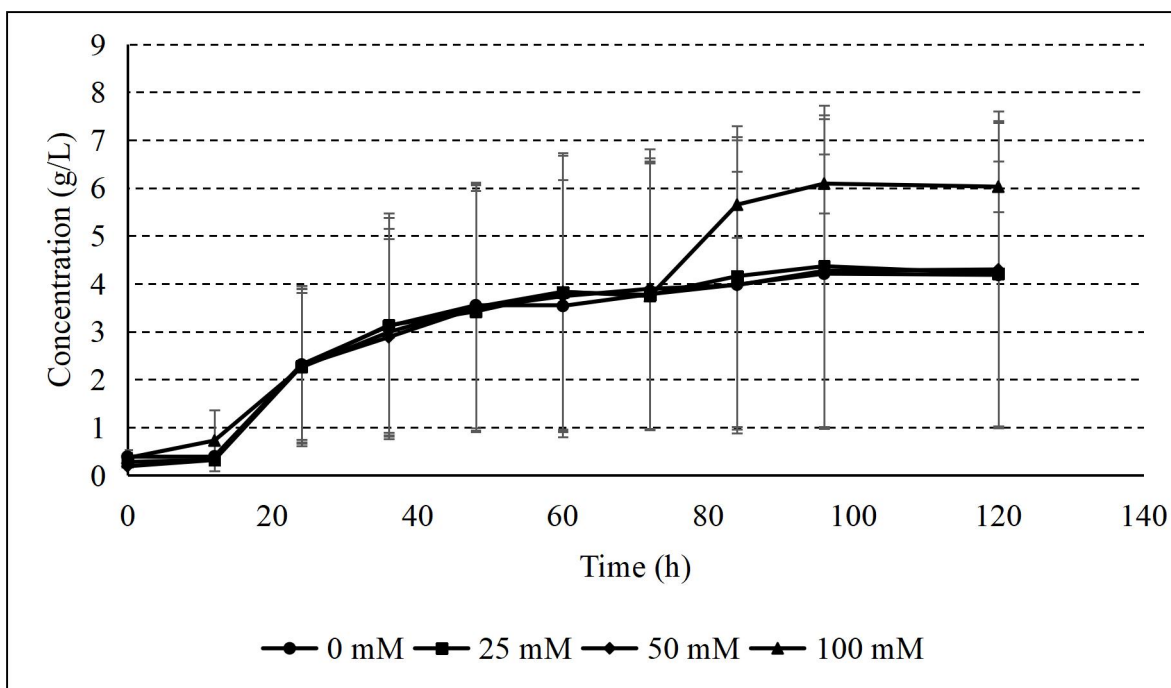


Figure 4.4: Time course of lactic acid concentration in broths containing TSEC with different strengths of citrate buffer during fed-batch bioethanol fermentation, as determined by HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

In addition to the lactic acid results, acetic acid production was also analysed during fermentation (Figure 4.5). The concentration of acetic acid in the non-buffered broth showed higher production compared to buffered broths. At 120 h, the maximum acetic acid production in the non-buffered broth was 0.99 g/L. Meanwhile, in 25, 50 and 100 mM, acetic acid increased steadily to final concentrations of 0.86, 0.83 and 0.80 g/L, respectively.

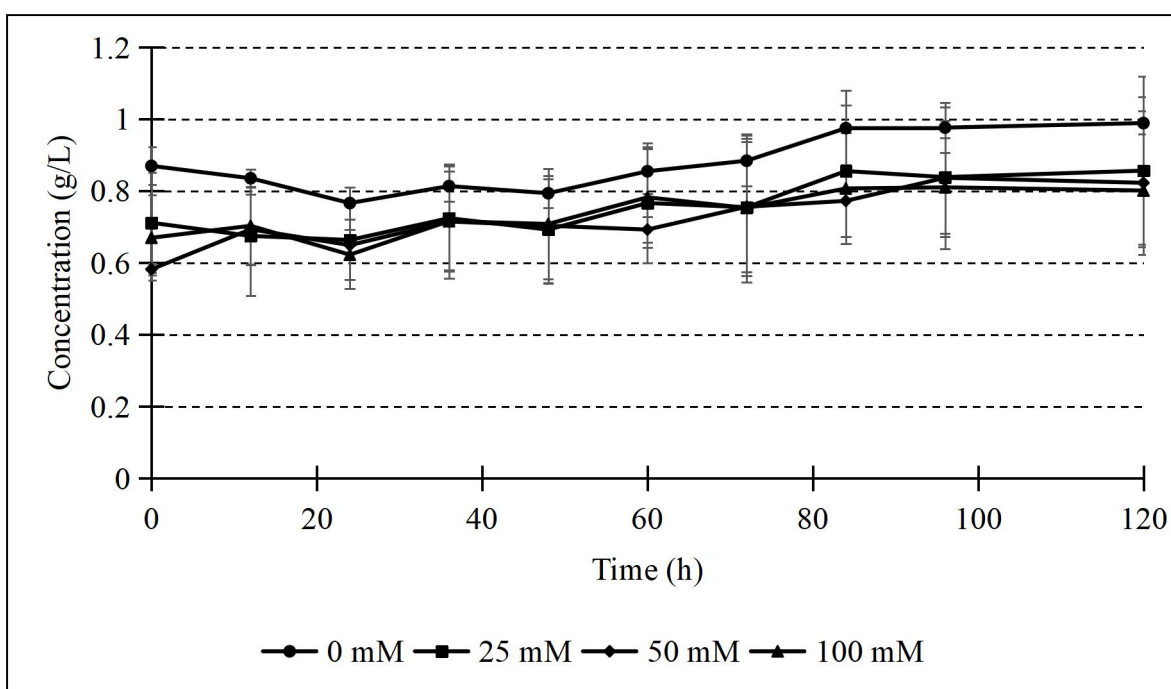


Figure 4.5: Time course of acetic acid concentration in broths containing TSEC with different strengths of citrate buffer during fed-batch bioethanol fermentation, as determined by HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

According to Table 4.3, the results had shown the non-buffered broth recorded the highest ethanol yields. In particular, the non-buffered broth highest ethanol yield with a concentration of 23.44 g/L (TEY of 62.94%) followed by samples from the 25 mM buffered broth with 23.22 g/L ethanol which equivalent to a theoretical yield of 62.33%. Ethanol production in 50 mM buffered broth showed reading with concentration of 20.67 g/L (TEY of 55.49%) and the lowest ethanol production was detected in 100 mM buffered broth as 20.61 g/L or 55.33% of theoretical yield.

Table 4.3: The maximum production and ethanol yield produced during fed-batch fermentation of bioethanol using Total Sago Effluent Concentrate (TSEC) of different buffer strengths.

Fermentation broth	Maximum production of ethanol (g/L)	Experimental theoretical yield (%)
Non-buffered	23.44 ± 4.17	62.94 ± 11.18
25 mM	23.22 ± 2.45	62.33 ± 6.57
50 mM	20.67 ± 2.85	55.49 ± 7.66
100 mM	20.61 ± 1.79	55.33 ± 4.82

*Data are mean triplicates ± S.D (Different superscript letters in the same column show significant differences.

CHAPTER 5

DISCUSSION

Ethanol is the by-product of the fermentation process of yeast with glucose or other fermentable sugars as the carbon source. Bioethanol derived from non-edible sources such as lignocellulosic biomass is important source of renewable energy. Furthermore, second generation bioethanol performs better compared to fossil fuels, both environmentally and in net energy output (Wiloso et al., 2012). However, advanced technologies are required for the production of second generation bioethanol prior to fermentation process (Tse et al., 2021). Many previous studies are currently reconduct to enhance bioethanol production, specifically in developing cost-efficient processes and higher bioethanol yield. High bioethanol production is important to achieve energy security to combat worldwide energy shortage (Azhar et al., 2017; Derman et al., 2018).

In the sago starch extraction industries, the production resulted in sago waste in the form of liquid and solid known as sago pith residue and sago effluent. These wastes can be found in abundant and are usually discarded into nearby streams. Treatments for these wastes could minimise industrial pollution however, it is unprofitable. One possible way to overcome this problem is by utilizing it as substrate for bioethanol production. According to Rijal (2020), sago waste contains 65.70% starch and 34.30% crude fibre which can be

used for bioethanol production. Sago waste, either in the form of wet solid, dry solid or effluent, contain carbohydrates in the form of lignocellulosic compounds. Compared to liquid waste, which readily dissolve into the environment, solid waste has greater carbohydrate content that can be broken down into glucose through hydrolysis. Study done by Rijal (2020) reported high bioethanol yield of 45.70% from the combination of wet solid hampas and *Saccharomyces cerevisiae*.

In this study, bioethanol was produced by utilizing Total Sago Effluent Concentrate via Simultaneous Saccharification and Fermentation (SSF) in the non-buffered broth against buffered broths with three different buffer strengths (25, 50 and 100 mM). Total Sago Effluent Concentrate (TSEC) is a term used to describe a high starchy solid component of Total Sago Effluent (TSE) which undergone physical pretreatment process of grinding and centrifugation. TSEC is composed of sago effluent hydrolysate and sago hampas. The SSF process is a process where both enzymatic hydrolysis and fermentation process occur simultaneously in one single step. Vincent et al. (2021) reported that SSF is a promising fermentation process in bioethanol production compared to Separate Hydrolysis and Fermentation (SHF) due to its higher reaction rates, higher ethanol yields and concentrations.

Initially, compositional analyses were done on the TSEC prior to SSF process. These analyses are important as implementation of the pretreatment process to saccharify the substrate relies on the composition of cellulose, hemicellulose and lignin on lignocellulosic biomass used (Tse et al., 2021). According to the results shown in Table 4.1, the water component of TSEC is 88.11% and 1.31% soluble starch. The remaining are solid components of TSEC. The dried solid portion contains 65.80% starch, 5.95% cellulose, 18.20% hemicellulose and 5.25% lignin (Table 4.2). These results showed lower fiber content (29.4%) compared to the findings by Hung et al. (2018) and Vincent et al. (2021) with fiber contents of 36.7% and 36.1%, respectively. The starch content in this study is higher than previous findings by Awg-Adeni et al. (2010) in the range of 30%-50%. These differences may be due to the mechanical pulverization and centrifugation process which had broken down the cellulose to hemicelluloses thus, resulted to low cellulose content, high hemicellulose content simultaneously contributed to high starch content in this study. Hence, these findings suggested that the TSEC is a suitable material for amylolytic hydrolysis and subsequently as substrate for ethanolic fermentation by fermenting microorganisms such as *S. cerevisiae* (Hung et al., 2018).

Enzymatic saccharification processes are crucial steps in yielding feasible amount of fermentable sugars for ethanolic fermentation by microorganisms. Enzymes are

important biocatalysts to convert polysaccharides to smaller fermentable saccharides (Vincent et al., 2021). In this study, α -amylase and glucoamylase were used as amylolytic enzymes to break down the starch in the TSEC into maltose and glucose prior to the fermentation process. These amyloytic enzymes consist of α -1,4 and α -1,6-debranching hydrolyses to degrade the complex carbohydrates in TSEC where they act synergistically during the successive enzymatic saccharification of starch (Hung et al., 2018). This process requires pH within the range of 4.8 to 5.5 which is usually maintained by adding 50 mM citrate buffer to the fermentation broth. However, the use of 50 mM citrate buffer for enzymatic hydrolysis is not feasible when commercial batches of fermentation are performed (Deshavath et al., 2021). Thus, in this study, the ethanolic fermentation via fed-batch SSF using the TSEC was conducted in different strengths of citrate buffer (25, 50 and 100 mM) and also without citrate buffer to observe the efficiency of enzymatic hydrolysis while simultaneously improves ethanol yield and productivity.

Thermotolerant yeasts and/or bacteria are required to achieve the SSF process. It is important for these microorganisms are compatible with both enzymatic and fermentation process despite temperature differences (Tse et al., 2021; Marulanda et al., 2019). Various microorganisms such as yeast, fungi and bacteria have been tested for their fermentation capacity and results vary greatly amongst microorganisms in terms of their ability to

produce ethanol. In this study, *S. cerevisiae* or the common baker's yeast was used as a fermenting agent. This yeast strain is synonymous to alcoholic fermentation due to its efficiency in generating and producing higher ethanol yield with more than 80% of theoretical yield (Derman et al., 2018; Parapouli et al., 2020). *S. cerevisiae* is also reported to have high tolerance towards high sugar concentrations and has ability to produce a number of aromatic and volatile compounds (Parapouli et al., 2020). As a Generally Recognized as Safe (GRAS) ethanologen, *S. cerevisiae* have been widely studied and reviewed for high ethanol tolerance and production, stress tolerance, flexibility in genetic improvement and effective adaptation for large scale fermentation. In the presence of glucose, *S. cerevisiae* is capable to grow in aerobic and anaerobic conditions. Under anaerobic conditions, *S. cerevisiae* produce acetaldehyde later reduced to ethanol (Krantz et al., 2004; Tse et al., 2021). It uses the glycolysis to catabolize sugars reaching the step of pyruvic acid formation. In follows, the latter is converted by pyruvate decarboxylase to acetaldehyde and carbon dioxide, which in turn is reduced to ethanol by alcohol dehydrogenase and releasing NAD⁺ at the same time. The terminal step reactions that lead to ethanol are therefore very important and constitute the basis for major fermentation industries (Parapouli et al., 2020). Industrial production of bioethanol production by *S.*

cerevisiae is considered economical and feasible as it grows rapidly which meets the specifications (Chang et al., 2018).

High Performance Liquid Chromatography (HPLC) analysis was conducted to analyse the presence of ethanol and carbohydrate from TSEC. At the beginning process of SSF, ethanol was produced at 12 h in all fermentation broths. According to the results in Figure 4.2, the highest production of ethanol at 12 h was by the non-buffered broth followed by 100, 25 and 50 mM of the buffered broths, with values of 1.19, 1.02, 0.92 and 0.87 g/L, respectively. The simultaneous conversion of glucose released from the TSEC to ethanol indicated SSF was successfully achieved as the procedure consumed a shorter duration. The glucose was consumed by *S. cerevisiae* during the first 12 h showed the ethanol concentration gradually increased while the glucose concentration decreased steeply. In this study, SSF was carried out in fed-batch fermentation mode whereby nutrients were supplemented to the fermentation broths throughout fermentation process to enhance ethanol yield (Tse et al., 2021). The result in Figure 4.2 showed ethanol production increased to its maximum production at 96 h indicated yeast alcohol production is maintained by consistent nutrients supply. In comparison to the study done by Hung et al. in 2018, the ethanol yield peaked at 48 h in ethanolic fermentation conducted via batch mode of SSF using sago hampas and *S. cerevisiae*. A study done by Ariff et al. (2017) also

reported that fed-batch fermentation is useful to reduce the risk of metabolic overflow which correlated to too high ethanol production and reduction of biomass yield. During ethanolic fermentation, *S. cerevisiae* produced 0.51 g ethanol from each gram glucose consumed and theoretically, depending on different processing methods, the ethanol yields varied from 64% to 90% (Ruchala et al., 2020; Guo et al., 2022). Statistical analysis was conducted using Tukey's *post hoc* test showed the glucose to ethanol conversion was about 62.94% of theoretical value which considered as fair yield. Figure 5.1 shows the theoretical ethanol yield from fermentations using different strengths of buffer with p-value <0.05. The overall data are presented in the Appendices. SPSS analysis was carried out to compare the ethanol yield of each broth tested. Figure 5.1 shows that there is no significant difference in all the fermentation broths.

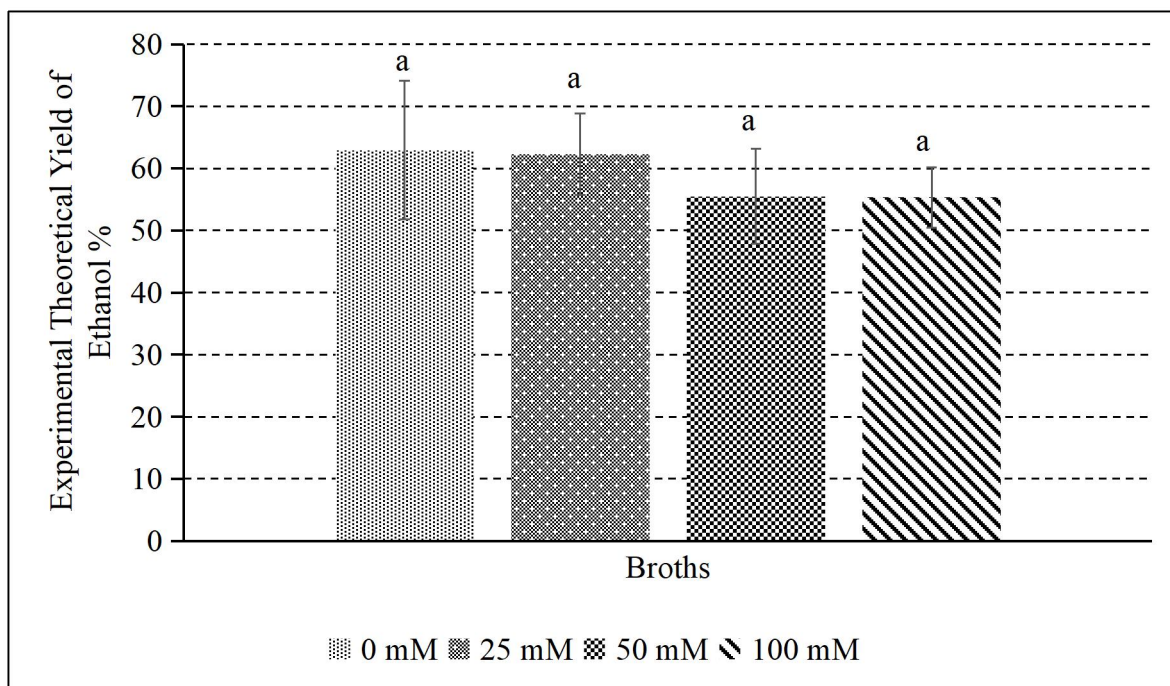


Figure 5.1: Experimental theoretical yield of bioethanol in different buffer strengths during fed-batch fermentation. [Note: Letters on top of column indicates statistically significant differences via Tukey's *post hoc* test, $\alpha=0.05$]

The initial glucose of all broths was recorded as the highest glucose concentrations indicating the efficiency of enzymatic hydrolysis of the TSEC. *S. cerevisiae* had consumed the glucose during the first 48 h showed a sharp decrease in glucose concentration in all fermentation broths. The SSF process was conducted in fed-batch fermentation mode whereby 5 g of TSEC and amylolytic enzymes were added into the fermentation broths every 24, 48 and 72 h. From Figure 4.3, it showed a slight raised of glucose concentration at 60 and 84 h suggesting the amylolytic enzymes successfully breakdown the starch to glucose in the fermentation broths. The glucose concentration gradually decreased after 84 h indicating the glucose presents in the fermentation broths were fermented into ethanol by

S. cerevisiae. However, residual sugar was detected in the broths (25 and 100 mM buffered broth) as it slowly depleted until the end of SSF which showed these sugars were not all converted to ethanol. In the non-buffered broth and 50 mM buffered broth, no glucose was detected at 120 h. It is possible that the remained sugars were in minimal quantities for HPLC to analyse. These observations were also reported by Hung et al. (2018), Vincent et al. (2021) and Assan et al. (2023), suggesting that there were presence of other hexose sugars other than glucose.

In this study, Phenol-sulfuric Acid (PSA) assay was also performed to complement the HPLC analyses that are limited in quantifying more complex saccharides such as oligosaccharides and short chain polysaccharides. According to Masuko et al. (2005), the PSA assay is the most accurate colourmetric method for carbohydrate estimation. From Figure 4.1, the results showed that there are residual carbohydrates detected in all fermentation broths at 120 h suggesting presence of other saccharides that are not consumed or fermentable to produce ethanol.

Lactic acid is one of the by-products from bioethanol production. The presence of this weak acid was monitored as this can be an indicator of contamination by lactobacilli (Vincent et al., 2021). It is possible that the abundance of lactobacilli in bioethanol process is due to its tolerance to ethanol, low pH and high temperature (30-45 °C) (Lucena et al.,

2010; Brexo & Sant'Ana, 2017). Disruption by lactobacilli also risks the growth of associated yeasts by competing to utilise available glucose and resulting in toxins production which can contaminate the fermentation broths. Based on the results presented in Figure 4.4, the highest lactic acid concentration was produced in 100 mM buffered broth (6.02 g/L) compared to the non-buffered broth, 25 and 50 mM buffered broths with concentration of 4.18, 4.20 and 4.30 g/L, respectively. High concentration of lactic acid can affect the efficiency of fermentation and reduce ethanol production. In fuel ethanol industry, sterile conditions are not generally practiced resulting in microbial contamination (Graves et al., 2006). These conditions can be inconvenient in industrial scale whereby facility operations need to be halt for cleaning and sterilization before next steps can be proceed (Tse et al., 2021). From the findings of in this study, lactic acid concentrations in the fermentation broths were detected in minimal amount thus, showing that the SSF was free of contamination.

Acetic acid is a key biomarker of biomass hydrolysis. According to Vincent et al. (2015a), an increase in acetic acid concentration during the SSF signifies continuous degradation of lignocellulosic materials. From Figure 4.5, the concentrations of acetic acid in the fermentation broths increased steadily until 120 h in the range 0.80 g/L (in 50 mM buffered broth) until 0.99 g/L (in non-buffered broth). According to Hung et al. in 2018,

elevation of acetic acid concentration is possibly due to the oxidation of ethanol during the SSF process. Moreover, the rate of acetic acid produced is correlated with the amount of fermentable sugars (Yang et al., 2010). During ethanolic fermentation process, minimal quantities of acetic acid produced by *S. cerevisiae* enhanced ethanol production rate. As reported by Graves et al. in 2017, the presence of acetic acid requires yeast cells to produce more ATP in order to maintain the internal pH and increases the glycolytic reflux, thereby stimulating the rate of ethanol production. However, acetic acid in high concentration can be toxic to the yeast as excess acetic acid amount will lead to the inhibition of cell growth and metabolism caused by the undissociated molecules of acetic acid that passively permeate through the plasma membrane when the pH of the surrounding environment is lower than pKa value (Graves et al., 2017; Ndukwe et al., 2019). According to Graves et al. (2006), ethanol production is completely inhibited when acetic acid concentration more than 0.80% w/v or 8 g/L. In this study, the acetic acid concentration detected is minimal thus, the production of ethanol was not affected.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Globally, bioethanol production is becoming more widespread as the demand for energy increases. Therefore, new technologies for bioethanol production are extensively explored. Advanced bioethanol production technology allows production from wide range of feedstock materials simultaneously enhancing ethanol yields. The Sarawak region in Malaysia is known to operate large sago starch production plants. This industry has resulted in the generation of wastes that are rich in organic materials. These wastes are often handled inefficiently by discarding into nearby watercourse which can lead to environmental issues. Thus, this research was done to assess the feasibility of converting the sago wastes, known as Total Sago Effluent Concentrate (TSEC), as a substrate to produce ethanol in buffered and non-buffered broth. Different buffer strengths of 25, 50 and 100 mM were tested and compared to the non-buffered broth.

In summary, the results showed that the formulation for the fermentation broths supported ethanol production and indicated there are no significance differences in all fermentation broths. Bioethanol production using the TSEC as substrate was recorded the

highest at 62.93% of theoretical ethanol yield (TEY) for the non-buffered broth. For buffered broth, highest TEY were recorded at 62.33%, 55.49% and 55.33% for 25, 50 and 100 mM respectively. These findings also showed that the non-buffered broth yielded more ethanol than the buffered broths. Thus, ethanolic fermentation using the non-buffered TSEC as feedstock is possible for bioethanol production via fed-batch Simultaneous Saccharification and Fermentation (SSF) with the aid of amylolytic enzymes and *Saccharomyces cerevisiae* to produce bioethanol.

6.2 Recommendations

The TSEC comprises of not only starch but, also cellulose, hemicellulose and lignin. In addition to the pretreatment process, enzymatic hydrolysis is fundamental in bioethanol production from lignocellulosic materials. For future studies, it is recommended to include cellulolytic enzyme during SSF process to optimize glucose conversion from cellulosic materials. The combination of amylolytic and cellulolytic enzymes may potentially enhanced TSEC conversion and bioethanol production yields. It is also recommended to investigate the efficiency of cellulose and glucose conversion in various buffer strengths. Additional compositional analyses using High Performance Liquid Chromatography

(HPLC) for other common carbohydrates such as arabinose, cellobiose and xylose can be done for future research.

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APPENDICES

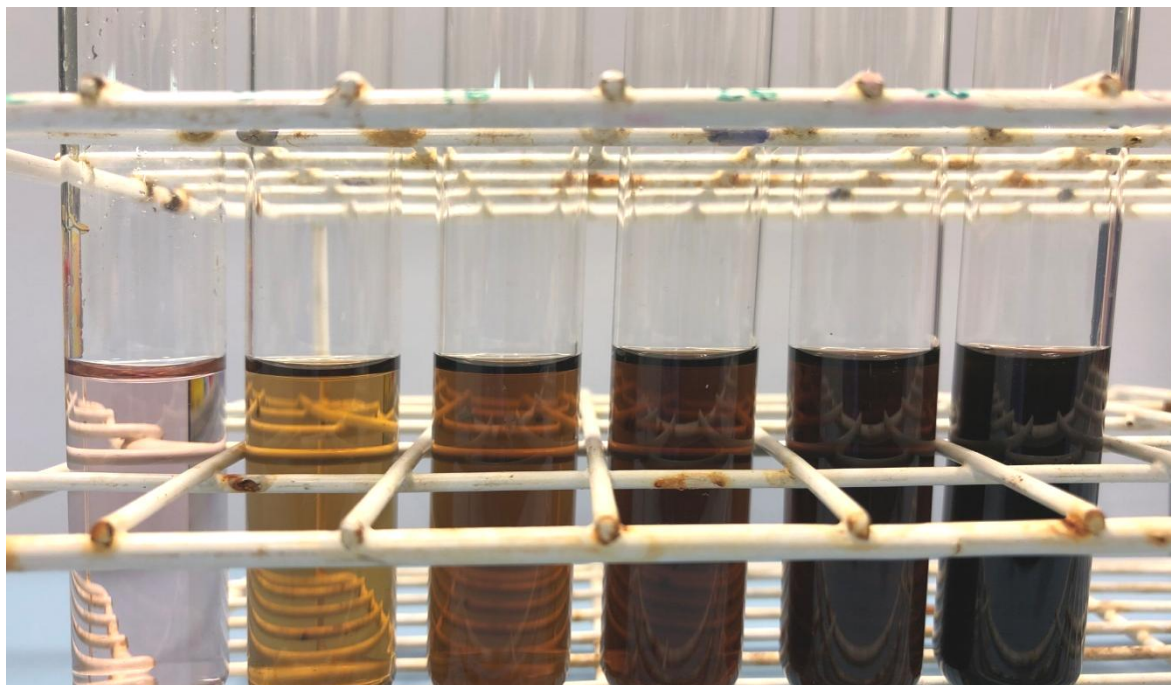


Figure 1: Colour development for standard curve of Phenol-sulfuric Acid assay.

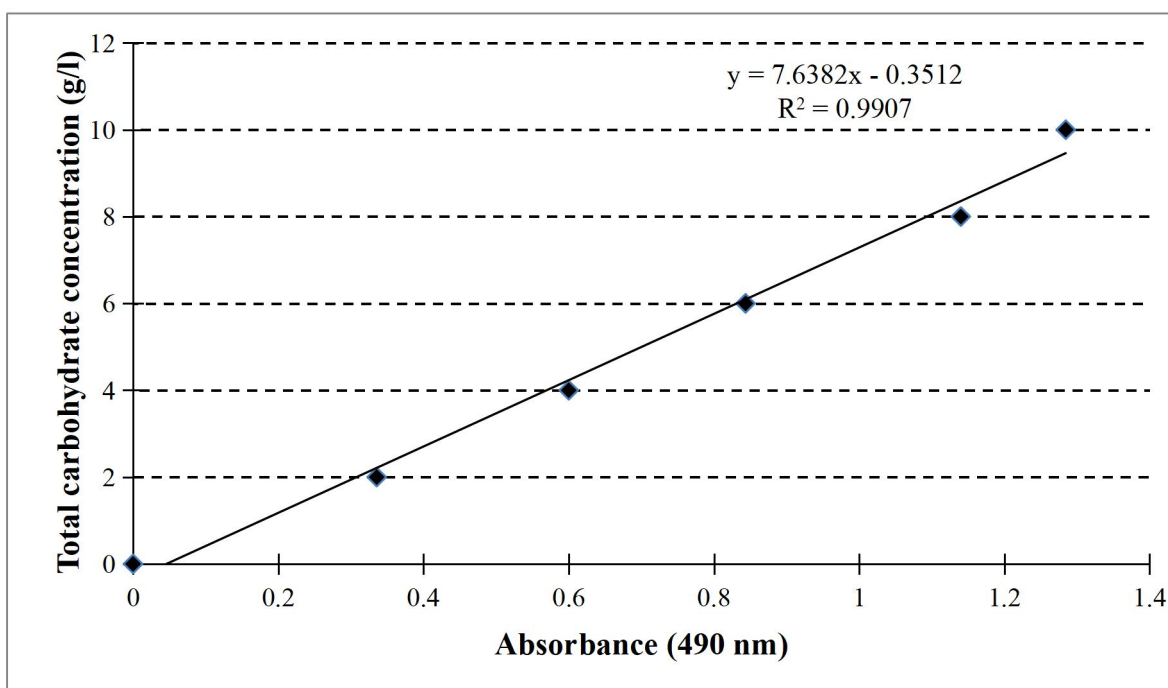


Figure 2: Standard curve of glucose for PSA.

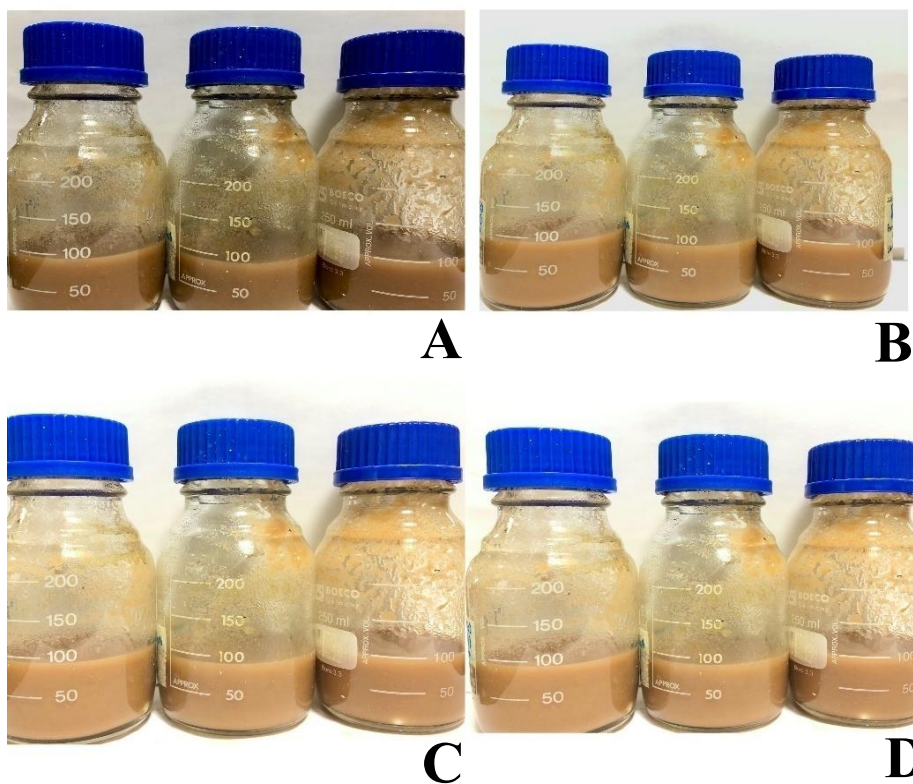
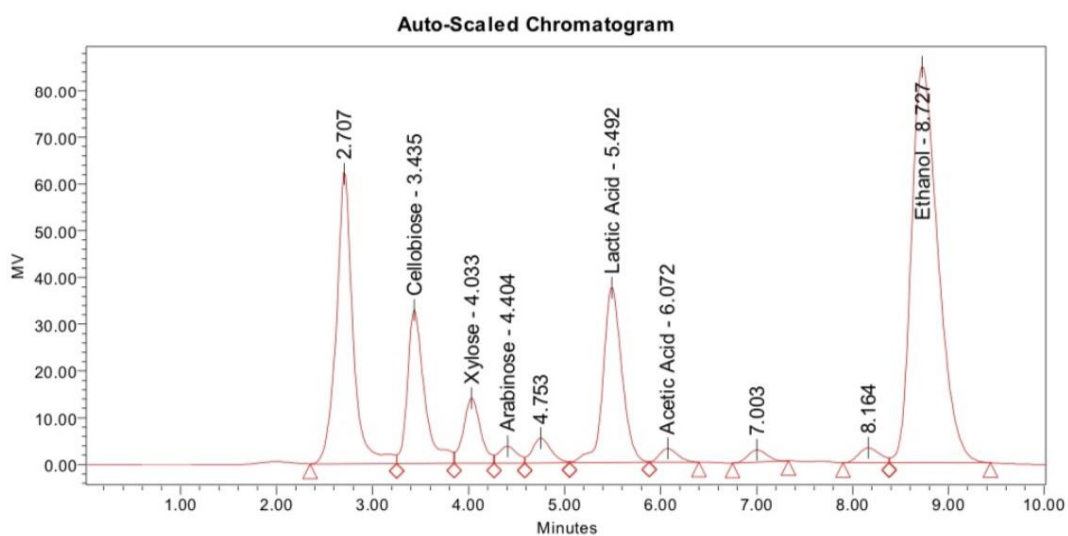


Figure 3: Fermentation Broths containing TSEC at 120 h (A) Non-buffered Broth; (B) 25 mM Broth; (C) 50 mM Buffered Broth; (D) 100 mM Buffered Broth.

SAMPLE INFORMATION			
Sample Name:	120HS4	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	EFFA
Vial:	20	Acq. Method Set:	Lignocellulosic Fermentation
Injection #:	1	Processing Method	
Injection Volume:	20.00 ul	Channel Name:	410
Run Time:	10.0 Minutes	Proc. Chnl. Descr.:	410
Date Acquired:	19/1/2022 2:08:44 PM MYT		
Date Processed:	19/1/2022 2:18:46 PM MYT		



Peak Results						
	Name	RT	Area	Height	Amount	Units
1		2.707	751614	62436		
2	Cellobiose	3.435	406401	32868		g/L
3	Glucose	3.820				
4	Xylose	4.033	171245	13937		g/L
5	Arabinose	4.404	46950	3588		g/L
6		4.753	70943	5281		
7	Lactic Acid	5.492	508452	37513		g/L
8	Acetic Acid	6.072	38203	2938		g/L
9		7.003	35691	2590		
10		8.164	49555	3176		
11	Ethanol	8.727	1639855	84866		g/L

Figure 4: The chromatogram of High Performance Liquid Chromatography.

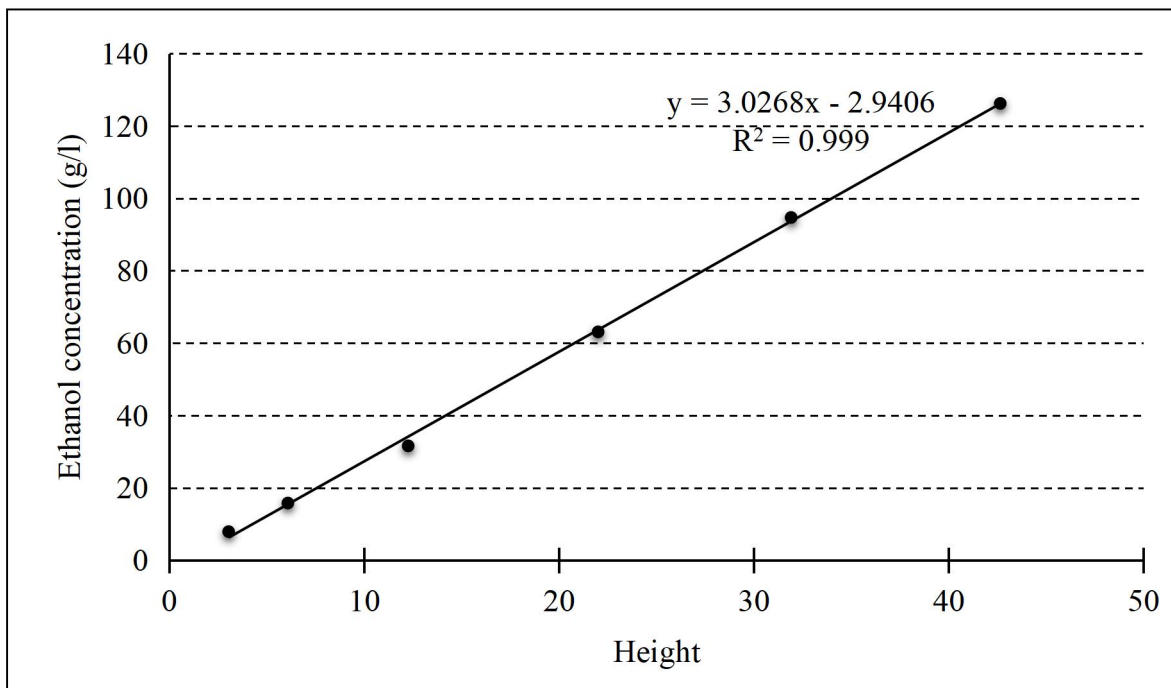


Figure 5: Standard curve for ethanol determination via HPLC.

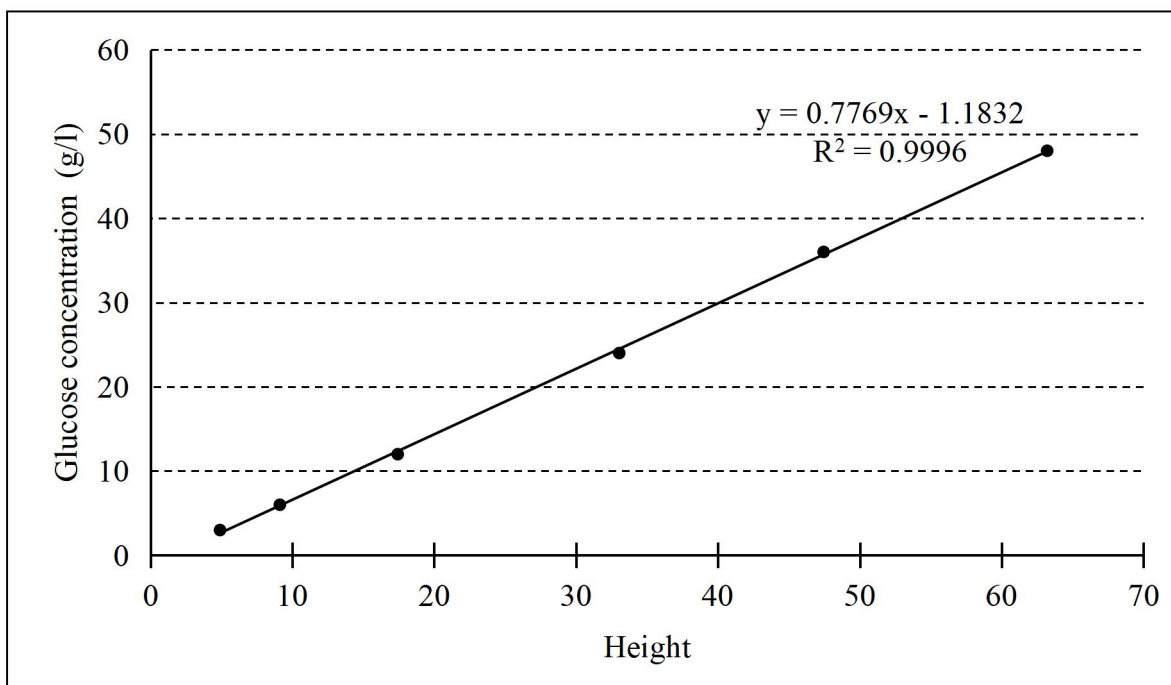


Figure 6: Standard curve for glucose determination via HPLC.

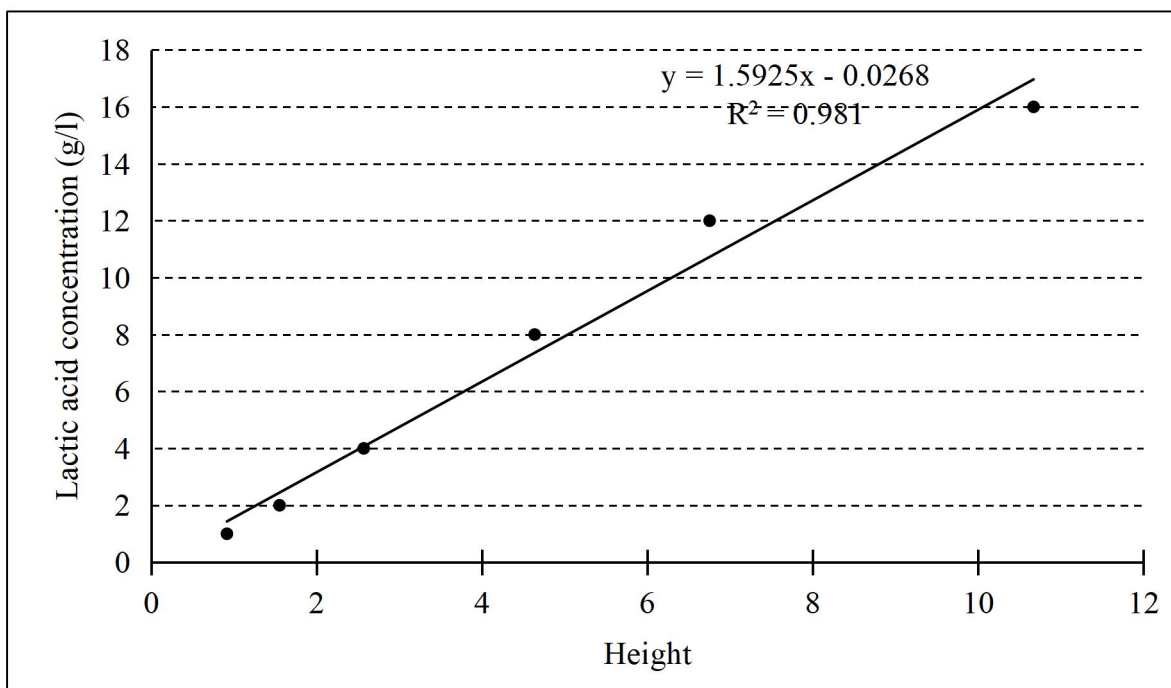


Figure 7: Standard curve for lactic acid determination via HPLC.

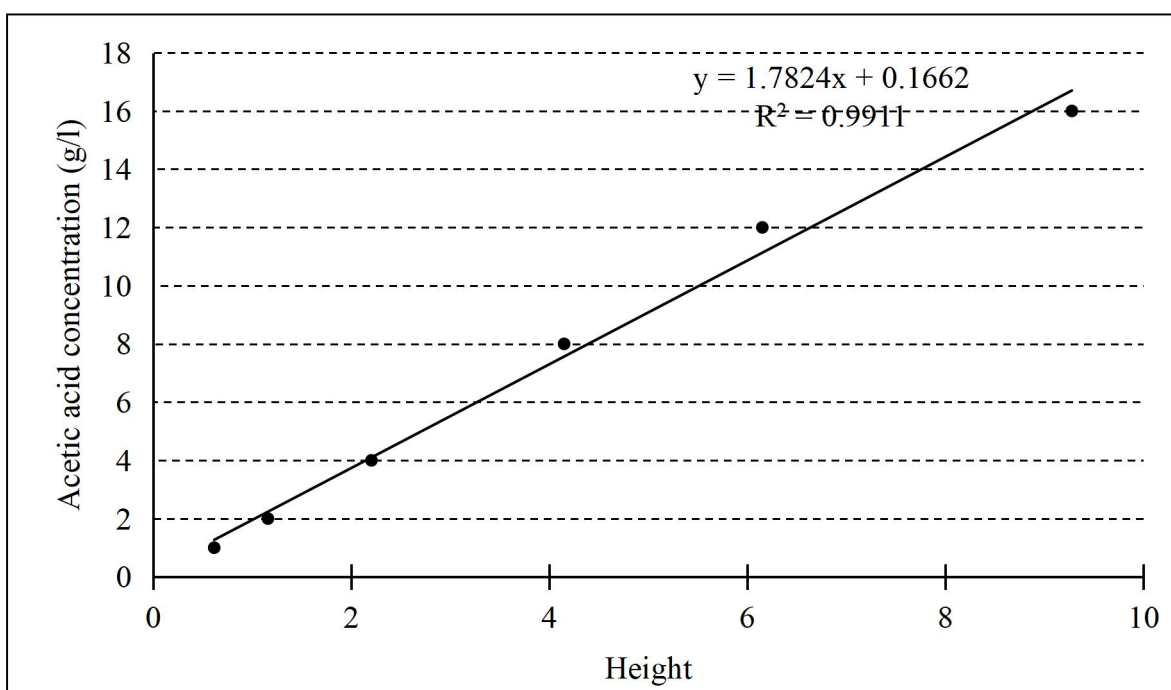


Figure 8: Standard curve for acetic acid determination via HPLC.

Table 1: Results of fermentation of non-buffered Total Sago Effluent Concentrate.

Hour (s)	Ethanol (g/L)	Glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0	0 ± 0	37.30 ± 1.49	0.39 ± 0.14	0.86 ± 0.05
12	1.19 ± 1.14	33.09 ± 1.42	0.39 ± 0.05	0.84 ± 0.02
24	10.52 ± 3.35	12.16 ± 0.82	2.31 ± 1.64	0.77 ± 0.04
36	16.69 ± 2.49	4.80 ± 0.47	3.12 ± 2.36	0.81 ± 0.04
48	18.12 ± 4.06	0 ± 0	3.55 ± 2.56	0.79 ± 0.04
60	20.29 ± 2.55	0.01 ± 0.02	3.54 ± 2.63	0.85 ± 0.06
72	21.79 ± 2.75	0 ± 0	3.78 ± 2.83	0.88 ± 0.07
84	22.25 ± 2.23	0.84 ± 1.46	3.98 ± 3.02	0.97 ± 0.11
96	23.44 ± 4.17	0 ± 0	4.21 ± 3.24	0.98 ± 0.07
120	22.39 ± 0.85	0 ± 0	4.18 ± 3.17	0.99 ± 0.13

Table 2: Results of fermentation of TSEC using 25 mM citrate buffer.

Hour (s)	Ethanol (g/L)	Glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0	0 ± 0	35.43 ± 1.12	0.28 ± 0.04	0.71 ± 0.14
12	0.92 ± 0.76	28.06 ± 3.79	0.32 ± 0.08	0.67 ± 0.17
24	9.61 ± 1.20	11.99 ± 0.99	2.28 ± 1.53	0.66 ± 0.11
36	15.03 ± 0.86	5.08 ± 2.79	3.13 ± 2.24	0.72 ± 0.14
48	17.06 ± 1.46	0 ± 0	3.42 ± 2.52	0.69 ± 0.15
60	19.69 ± 2.79	0 ± 0	3.82 ± 2.86	0.77 ± 0.17
72	18.79 ± 1.00	0 ± 0	3.77 ± 2.79	0.75 ± 0.19
84	21.87 ± 1.48	0.79 ± 0.61	4.16 ± 3.14	0.86 ± 0.18
96	23.22 ± 2.45	0.47 ± 0.82	4.37 ± 3.36	0.84 ± 0.15
120	22.18 ± 0.72	0.39 ± 0.68	4.21 ± 3.19	0.86 ± 0.21

Table 3: Results of fermentation of TSEC using 50 mM citrate buffer.

Hour (s)	Ethanol (g/L)	Glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0	0 ± 0	31.31 ± 3.73	0.19 ± 0.01	0.58 ± 0.02
12	0.87 ± 0.79	30.62 ± 1.24	0.32 ± 0.06	0.69 ± 0.09
24	8.42 ± 1.55	11.99 ± 1.19	2.29 ± 1.60	0.64 ± 0.12
36	13.49 ± 0.73	6.59 ± 4.11	2.88 ± 2.04	0.72 ± 0.16
48	16.62 ± 2.10	1.52 ± 2.64	3.49 ± 2.57	0.70 ± 0.16
60	16.58 ± 5.67	0.90 ± 1.19	3.74 ± 2.93	0.69 ± 0.04
72	17.88 ± 1.55	0 ± 0	3.89 ± 2.92	0.76 ± 0.18
84	18.06 ± 4.02	1.80 ± 1.57	3.97 ± 3.09	0.77 ± 0.09
96	20.67 ± 2.85	0 ± 0	4.27 ± 3.26	0.84 ± 0.19
120	19.84 ± 2.46	0 ± 0	4.29 ± 3.31	0.82 ± 0.20

Table 4: Results of fermentation of TSEC using 100 mM citrate buffer.

Hour (s)	Ethanol (g/L)	Glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0	0 ± 0	33.05 ± 1.99	0.36 ± 0.12	0.67 ± 0.12
12	1.01 ± 0.96	32.03 ± 2.74	0.73 ± 0.64	0.70 ± 0.11
24	7.65 ± 2.27	12.43 ± 0.99	2.26 ± 1.64	0.62 ± 0.07
36	12.62 ± 1.85	10.23 ± 1.66	2.99 ± 2.17	0.72 ± 0.14
48	16.24 ± 1.53	1.64 ± 1.79	3.51 ± 2.58	0.71 ± 0.15
60	17.72 ± 2.69	2.47 ± 3.88	3.83 ± 2.90	0.78 ± 0.14
72	17.55 ± 0.96	0.75 ± 1.30	3.74 ± 2.78	0.75 ± 0.21
84	18.86 ± 3.03	2.26 ± 3.05	5.65 ± 0.69	0.81 ± 0.15
96	20.61 ± 1.79	1.43 ± 2.48	6.09 ± 0.62	0.81 ± 0.14
120	19.60 ± 0.39	0.54 ± 0.93	6.03 ± 0.53	0.80 ± 0.16

Table 5: *Post hoc* tests between four independent experiments using TSEC as substrate.

(I) Parameter	(J) Parameter	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Non-buffered	25 mM	.198893	2.094663	1.000	-6.50896	6.90674
	50 mM	2.808087	2.094663	.565	-3.89976	9.51594
	100 mM	2.503973	2.094663	.646	-4.20388	9.21182
25 mM	Non-buffered	-.198893	2.094663	1.000	-6.90674	6.50896
	50 mM	2.609193	2.094663	.618	-4.09866	9.31704
	100 mM	2.305080	2.094663	.699	-4.40277	9.01293
50 mM	Non-buffered	-2.808087	2.094663	.565	-9.51594	3.89976
	25 mM	-2.609193	2.094663	.618	-9.31704	4.09866
	100 mM	-.304113	2.094663	.999	-7.01196	6.40374
100 mM	Non-buffered	-2.503973	2.094663	.646	-9.21182	4.20388
	25 mM	-2.305080	2.094663	.699	-9.01293	4.40277
	50 mM	.304113	2.094663	.999	-6.40374	7.01196

*. The mean difference is significant at the 0.05 level