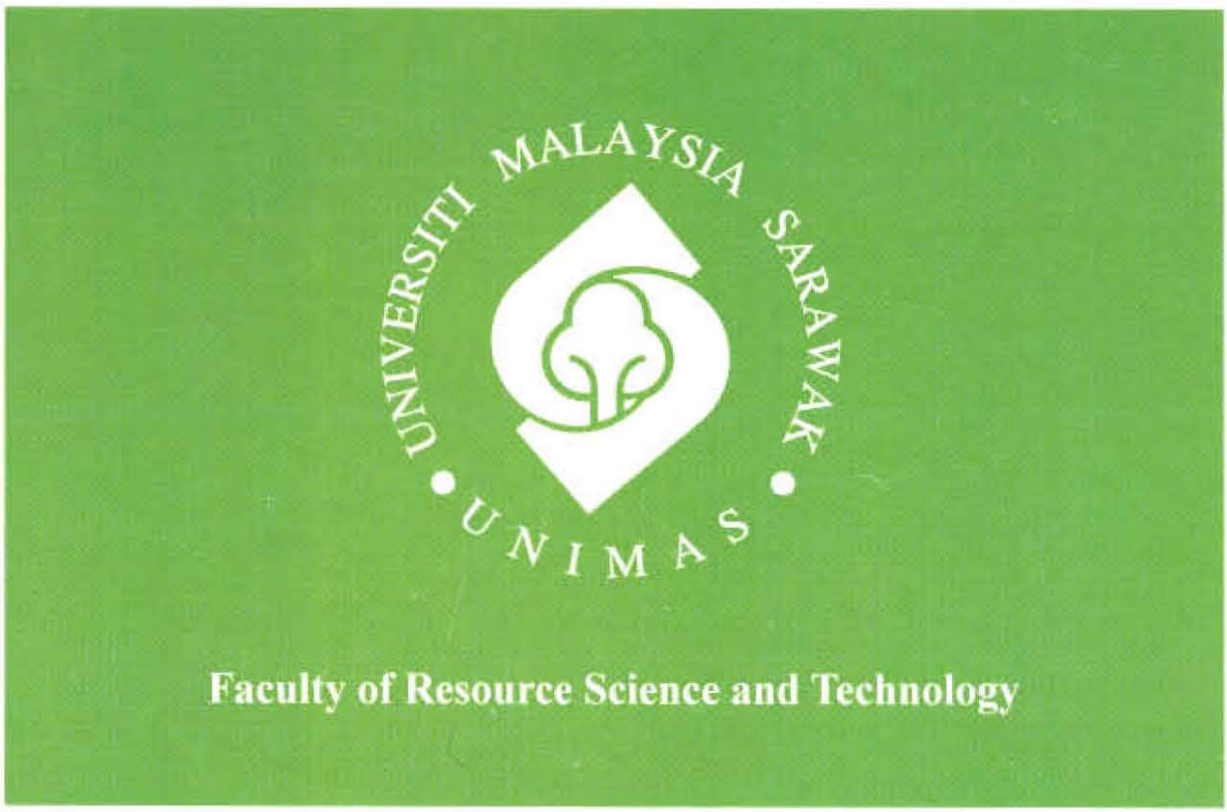


Potentials of Sago Fibre Hydrolysate (SFH) as the Sole Fermentation Medium for Production of Bioethanol

Sharifah Binti Mohammad

Master of Science
2018

Pusat Khidmat Maklumat Akademik
UNIVERSITI MALAYSIA SARAWAK

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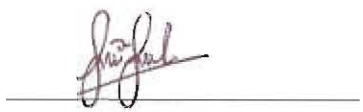
A thesis submitted

In fulfilment of the requirements for the degree of Master of Science (Biochemistry)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2018

DECLARATION

The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



Sharifah Binti Mohammad

Matric No: 15020152

Date: 30th January 2018

ACKNOWLEDGEMENT

Alhamdulillah, all praises to Allah SWT, the Almighty for this golden opportunity, studying Msc. under the supervision of Dr. Dayang Salwani Binti Awang Adeni, Prof. Dr. Kopli Bin Bujang and Dr Micky Vincent for their valuable supervision, scholarly inputs and consistent encouragement I received throughout this research work, altogether with my thesis writing. Your unconditional support and patience throughout this project has been most comforting through my difficult times. A person whom always made her/himself available to clarify my doubts despite the entire busy schedule and I am very proud to have the opportunity to learn under your research expertise in completion of my research programme.

I would like to extend with much appreciation of the crucial role of all Biochemistry Laboratory staffs in the Faculty of Resource Science and Technology, UNIMAS, especially Assoc. Prof. Cirilo Nolasco, Dr Fathurrahman and Mr Amin Manggi for their assistances. Plus, some faculty members have been very kind enough to extend their help, and I do hereby acknowledge all of them. Special thanks to my research mates and friends; Nadia, Farahin, Fara, Nurulamira, Helmi, Shamil, Zakaria, Faezzah, Ana, Ain, Fasha, Hafida, Rinie, Mila, Aimi, Hemnaarth, Syahir and Enry who helps me to assemble the parts and gave suggestions as well as encouragement upon completion of my research.

Finally, utmost thanks to my beloved mother, Mrs Hasnah Binti Ismail, my late father, Mr. Mohammad Bin Romli and all my siblings, for their steadfast support, understanding and sacrifices in making this journey reached its destination. I would like to acknowledge Tun Openg Research Chair (F07 (ORC29)/1235/2015 (16)) for funding this project.

ABSTRACT

Sago waste water which contains starchy fibres from sago starch processing mills commonly discharged directly to nearby stream thus contribute to serious environmental pollution. This study is an effort to utilize residual starch trapped in sago fibres as raw materials for bioethanol production, hence could minimize the amount of sago wastes. Sago fibre (SF) was utilized into Sago Fibre Hydrolysate (SFH) via enzymatic hydrolysis using commercial enzymes; Liquozyme SC DS and Spirizyme Fuel HS. The load of Sago Fibre Powder (SFP) at 7% (w/v) was used for the hydrolysis process. Lower glucose concentration (46.24 ± 2.27 g/L) was obtained, thus, an alternative method (cycles I, II, and III) which involved reusing the hydrolysate for subsequent enzymatic hydrolysis cycles was adopted. Greater improvement of higher glucose concentration (149.36 ± 1.53 g/L) was achieved after completing cycle III of the hydrolysis process. Glucose (92%) was found to be the highest monomeric sugar component in the hydrolysate, followed by cellobiose (3%), and other oligosaccharides (5%). This study confirmed that 50 g/L glucose from SFH media (without addition of nitrogen source) capable of generating maximum ethanol concentration at 20.33 ± 0.15 g/L, during 24 hrs of fermentation. Similar concentration of bioethanol was obtained in 50 g/L glucose of CG media (without addition of nitrogen source) which is at 20.04 ± 0.06 g/L. When SFH amended with YE, ethanol concentration was slightly higher (22.71 ± 0.31 g/L), by 2.38 g/L or 10.5%. However, ethanol concentration was much higher in SFH amended with Peptone (P) at 24.02 ± 0.01 g/L, whereas 0.50 ± 0.002 g/g of ethanol yield was generated. Thus, 97.16 ± 0.402% of fermentation efficiency was achieved during 24 hours of fermentation. In this study, ammonium sulfate, peptone and urea were found to be feasible as nitrogen source in bioethanol production by using CBY. Based on statistical analysis, ammonium sulfate (AS) was the best nitrogen source, give the highest ethanol yield

(0.50 ± 0.002 g/g) and highest fermentation efficiency in converting glucose to ethanol (98.58 ± 0.449%), followed by peptone (0.50 ± 0.002 g/g), with fermentation efficiency of 97.16 ± 0.402%, and urea (0.49 ± 0.002 g/g), with 96.34 ± 0.406% of fermentation efficiency. Conclusively, the glucose in sago fibre hydrolysate (SFH) was metabolized efficiently by CBY during ethanol fermentation, thus exhibits the capability of SFH to be an alternative substrate with less expensive nitrogen source for the renewable bioethanol production.

Keywords: Sago fibre, sago fibre powder, sago fibre hydrolysate, glucose, bioethanol, nitrogen source, ethanol yield, fermentation efficiency, commercial baker's yeast

Potensi Hidrolisat Hampas Sagu sebagai Medium Fermentasi Tunggal untuk Penghasilan Bioetanol

ABSTRAK

Air sisa sagu yang mengandungi serat kanji daripada kilang pemprosesan kanji sagu biasanya dibiarkan mengalir terus ke sungai berdekatan, justeru menyumbang kepada pencemaran alam sekitar yang amat serius. Kajian ini adalah satu usaha untuk menggunakan lebihan kanji yang terperangkap di dalam hampas sagu (SF) sebagai substrat bagi penghasilan bioetanol, dan secara tidak langsung meminimumkan jumlah sisa sagu yang dibuang. Hampas sagu (SF) digunakan bagi menghasilkan hidrolisat hampas sagu (SFH), dengan menggunakan kaedah hidrolisis berenzim menggunakan enzim komersil; Liquozyme SC DS dan Spirizyme Fuel HS. Kepekatan serbuk hampas sagu (SFP) pada 7% (w/v) digunakan bagi proses hidrolisis berenzim. Walaubagaimanapun, kepekatan glukosa yang diperolehi adalah rendah (46.24 ± 2.27 g/L), oleh itu, kaedah alternatif (kitaran I, II, dan III) yang melibatkan penggunaan semula hidrolisat daripada hidrolisis pertama untuk kegunaan hidrolisis seterusnya telah digunakan. Didapati kepekatan glukosa dalam hidrolisat telah meningkat kepada (149.36 ± 1.53 g/L), berjaya diperolehi setelah tamat kitaran III semasa proses hidrolisis. Glukosa (92%), didapati sebagai komponen gula monomer tertinggi di dalam hidrolisat, diikuti oleh sellobiosa (3%), dan lain- lain oligosakarida (5%). Kajian ini mengesahkan bahawa 50 g/L glukosa daripada media SFH (tanpa penambahan sumber nitrogen) mampu menghasilkan etanol pada kepekatan maksimum 20.33 ± 0.15 g/L, setelah 24 jam fermentasi. Kepekatan bioethanol yang sama turut diperolehi di dalam 50 g/L glukosa daripada media glukosa komersil (CG) (tanpa penambahan sumber nitrogen) pada 20.04 ± 0.06 g/L. Apabila SFH ditambah dengan ekstrak yis (YE), kepekatan etanol adalah sedikit lebih tinggi (22.71 ± 0.31 g/L), iaitu sebanyak 2.38 g/L atau 10.5% penambahan. Walaubagaimanapun,

kepekatan etanol jauh lebih tinggi di dalam SFH media yang ditambah dengan pepton (P), iaitu sebanyak 24.02 ± 0.01 g/L, dengan nisbah hasil penukaran substrat kepada produk ialah 0.50 ± 0.002 g/g, manakala nilai kecekapan fermentasi adalah 97.16 ± 0.402 %, setelah 24 jam fermentasi. Dalam kajian ini, ammonium sulfat, pepton dan urea didapati sebagai sumber nitrogen yang murah, dan sesuai digunakan oleh CBY untuk penghasilan bioetanol. Berdasarkan analisis statistik, ammonium sulfat (AS) adalah sumber nitrogen yang terbaik, dengan nisbah hasil penukaran substrat kepada produk (0.50 ± 0.002 g/g) dan kecekapan fermentasi tertinggi dalam menukarkan glukosa kepada etanol (98.58 ± 0.449 %), diikuti pepton (0.50 ± 0.002 g/g) dengan nilai kecekapan fermentasi 97.16 ± 0.402 %, dan urea (0.49 ± 0.002 g/g), dengan kecekapan fermentasi sebanyak 96.34 ± 0.406 %. Secara kesimpulannya, glukosa yang terhasil bersama-sama hidrolisat hampas sagu (SFH) dapat digunakan oleh CBY secara efisien ketika proses fermentasi bioetanol, menunjukkan keupayaan SFH sebagai substrat alternatif apabila digunakan bersama sumber nitrogen yang lebih murah untuk penghasilan bioetanol yang boleh diperbaharui.

Kata kunci: *Hampas sagu, serbuk hampas sagu, hidrolisat hampas sagu, glukosa, bioetanol, sumber nitrogen, hasil bioetanol, kecekapan fermentasi, yis komersil*

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
α	Alpha
AS	Ammonium Sulfate
Bioethanol	Biological Ethanol
cm	Centimeter
CBY	Commercial Bakers' Yeast
CG	Commercial Glucose
°C	Degree Celsius
DOA	Department of Agriculture
DNS	Dinitrosalicyclic Acid
DCW	Dry Cell Weight
E_f	Fermentation efficiency
g	Gram
g/g	Gram per gram
g/L	Gram per liter
g/Lhr	Gram per liter hour

HPLC	High Performance Liquid Chromatography
hr	Hour
kg	Kilogram
LCDA	Land Custody of Development Authority
L	Liter
µL/g	Microliter per gram
mg/L	Milligram per litre
mL	Milliliter
nm	Nano-meter
OD	Optical Density
P	Peptone
%	Percentage
KH_2PO_4	Potassium dihydrogen phosphate
PDA	Potato Dextrose Agar
Q_p	Product Volumetric Productivity
$Y_{p/s}$	Product yield based on substrate
x g	Times gravity
RM	Ringgit Malaysia
SF	Sago Fibre

CHAPTER 1

INTRODUCTION

1.1 Introduction

Lately, our world faces continuous depletion of its energy resources mainly based on non-renewable fuels and there has been increasing demands on the worldwide interest in alternative sources of renewable energy, particular in biofuel (Lin and Tanaka, 2006; Sanchez and Cardona, 2008). Biofuels which are derived from lignocellulosic solid biomass, liquid fuels and other biogases are the most rapidly growing renewable energy technologies. Currently research focused on the utilization of biomass waste for general fuel production, including bioethanol. Bioethanol is a promising alternative source, which is both renewable and environmental friendly liquid biofuel (Vincent *et al.*, 2015). Previously, bioethanol can be used either as a fuel additive or as a replacement of conventional non- renewable fuel (Tortora *et al.*, 2010). In fact, several countries have started to use bioethanol as their renewable fuel such as in USA, Brazil, China, Pakistan and India (Rubio-Arroyo *et al.*, 2005). Most of the source of bioethanol in Brazil is derived from sugarcane and corn whereas in the United States, vehicles can use E85; mixture of 15% gasoline and 85% ethanol (Tortora *et al.*, 2010).

In Malaysia, the State of Sarawak is recognized as the largest sago- growing areas, which is currently the world's biggest exporter of sago starch, exporting annually about 44, 000 t of starch mainly to peninsular Malaysia, Japan, Singapore and other countries (Awg- Adeni *et al.*, 2010). It has been reported that producing 1 ton of sago starch also releases 20 ton of wastewater, which containing about 5% (1 ton) sago fibre or sago fibre (Bujang *et al.*, 1996). Approximately, 60,000 tonnes of sago fibre are produced daily from eight sago starch

processing mills in Mukah (Manan *et al.*, 2003). In fact, wastes from the sago starch extraction process are being washed off into nearby rivers or deposited in the factory's compound every day. This condition potentially deteriorates the rivers and the environment (Bujang, 2015). Thus, in this study, the general idea is to maximise the utilization of sago fibre by enzymatic hydrolysis into glucose and other fermentable sugars as an alternative substrate for bioethanol production, which concomitantly assisting in managing sago wastes generated by sago factories.

Sago fibre, which is free and abundant - is derived as a starchy lignocellulosic by-product produced from extraction of starch from the pith of the sago palm. On a dry basis, sago fibre contains about 58% of residual starch together with high content of cellulosic components (Linggang *et al.*, 2012). According to Lin and Tanaka (2006), most of the agricultural wastes containing starch plays a vital role in ethanol fermentation by microbial processes. Previously, the sugar hydrolysate gotten from enzymatic hydrolysis of sago fibre has been revealed to be feasible as a cheap substrate for ethanol fermentation using commercial baker's yeast (Awg-Adeni *et al.*, 2013). Some studies on the exploitation of sago fibre have also been described elsewhere for animal feed, confectionery syrup, compost for mushroom cultivation, and raw material for locally isolated microbes to generate fermentable sugars and enzymes (Apun *et al.*, 2000; Rifat *et al.*, 2003; Shahrim *et al.*, 2008; Janggu and Bujang, 2009; Linggang *et al.*, 2012). Commonly, ethanol production via fermentation route comprises a series of biochemical reactions of simple sugars (i.e. glucose, fructose, sucrose, etc.) by yeast or bacteria (Chin and H'ng, 2013). Most ethanol related industries used *Saccharomyces cerevisiae* or commercial baker's yeast to ferment glucose into ethanol.

Besides, supplementing nitrogen sources such as yeast extract, peptone, urea and ammonium sulfate could enhance the ethanol concentration, ethanol yield and improved fermentation

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Sago fibre, which is free and abundant - is derived as a starchy lignocellulosic by-product produced from extraction of starch from the pith of the sago palm. On a dry basis, sago fibre contains about 58% of residual starch together with high content of cellulosic components (Linggang *et al.*, 2012). According to Lin and Tanaka (2006), most of the agricultural wastes containing starch plays a vital role in ethanol fermentation by microbial processes. Previously, the sugar hydrolysate gotten from enzymatic hydrolysis of sago fibre has been revealed to be feasible as a cheap substrate for ethanol fermentation using commercial baker's yeast (Awg-Adeni *et al.*, 2013). Some studies on the exploitation of sago fibre have also been described elsewhere for animal feed, confectionery syrup, compost for mushroom cultivation, and raw material for locally isolated microbes to generate fermentable sugars and enzymes (Apun *et al.*, 2000; Rifat *et al.*, 2003; Shahrim *et al.*, 2008; Janggu and Bujang, 2009; Linggang *et al.*, 2012). Commonly, ethanol production via fermentation route comprises a series of biochemical reactions of simple sugars (i.e. glucose, fructose, sucrose, etc.) by yeast or bacteria (Chin and H'ng, 2013). Most ethanol related industries used *Saccharomyces cerevisiae* or commercial baker's yeast to ferment glucose into ethanol.

Besides, supplementing nitrogen sources such as yeast extract, peptone, urea and ammonium sulfate could enhance the ethanol concentration, ethanol yield and improved fermentation

efficiency as well (Laopaiboon *et al.,* 2009). Yeast extract would assist in promoting cell growth, whereas ammonium salts help to stimulate glucose fermentation by lowering induction period and maintaining an optimum carbon to nitrogen (C/N) ratio. The addition of urea promotes specific growth rate, ethanol tolerance, increased in ethanol yield and lowered the formation of by-products (Jorgensen, 2009). Trace elements, such as zinc is important for stimulating cell growth and cell metabolism for all organisms (Zhao *et al.,* 2009) and without Zn, the fermentation process becomes slow (Russell, 2003). Magnesium has shown to sustain healthy cells by providing intracellular magnesium ion transfer at higher level (Ciesarova *et al.,* 1996). Calcium supplementation is mainly to augment the growth of yeast cell (Russell, 2003).

Despite all these factors, ethanol production by fermentation process always been affected by the high costs of raw materials. The research into new and alternative carbon sources such as industrial by- products and agricultural residues for the use in bioethanol fermentation is needed to sustain for the successful development of fermentation industries against traditional grain-based processes. A renewable raw material, which is locally abundant is considered as an excellent feedstock for bioethanol production due to high carbohydrate content. In any ethanol fermentation process, the greater substrate load would lead to increase ethanol concentration, and therefore improve the efficiency of downstream processing. According to Awg-Adeni et al. (2013), hydrolysate of sago fibre cannot be promoted as the sole fermentation media if higher concentration of glucose (>100 g/L) was employed. Thus, in this study, lower concentration of glucose was implemented to obtain maximum ethanol concentration.

This project emphasizes on the utilization of sago fibre which known to be a local waste with high carbohydrate content as an alternative substrate prior for production of ethanol via

microbial fermentation. However, the addition of other nitrogen sources into fermentation media is required to improve the growth of baker's yeast and enhance the ethanol fermentability towards commercial baker's yeast.

1.2 Objectives

The general objective of this study is to examine the feasibility of SFH as a sole fermentation media for production of bioethanol by using commercial baker's yeast (CBY).

The specific objectives of this project are to:

i. Analyse the ethanol fermentability utilizing SFH at initial glucose of 50 g/L and 80 g/L without addition of any nitrogen source.

ii. Identify the most suitable nitrogen sources (yeast extract, peptone, ammonium sulfate and urea) to be supplemented with sago fibre hydrolysate (SFH) containing 50 g/L and 80 g/L glucose for maximum generation of ethanol.

iii. Evaluate the overall ethanol fermentation performance between sago fibre hydrolysate (SFH) and commercial glucose (CG) with respect to the yield, ethanol productivity and fermentation efficiency.

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CHAPTER 2

LITERATURE REVIEW

2.1 Sago Palm *(Metroxylon sagu)*

2.1.1 Sago Palm and Cultivation

Sago palm or scientifically known as *Metroxylon sagu* (Figure 1) is found in abundance in the South East Asian region, particularly in Sarawak and most parts of Indonesia where this palm has been known to be used as a food commodity for hundreds of years. In the 19th century, sago starch served as the main food source for the local inhabitants of the Malay Archipelago region (Abd-Aziz, 2002). In Sarawak, large sago estates are found in Mukah, with small scale cultivation scattered all over the state (Bujang, 2015).



Figure 1: Sago Palm *(Metroxylon sagu)* in Sarawak (photo credit: to K. B. Bujang)

The sago palm is known to be able to thrive in acidic peat soils and harsh swampy areas, where only very few other crops survived (Kiew, 1977; Karim *et al.*, 2008). The palm is an extremely hardy plant which could grow well with minimum care apart from some ground clearing during the first stage of its growth and known to be robust without any serious diseases. Apart from these, sago palm is also reported to be unaffected to floods, drought, fire and strong winds (Chew *et al.*, 1999; Bujang, 2006). Although the first waiting period is long (can be up to 10 years for the plant to be harvestable), sago is the highest starch producer at 25 tons/ha/year, 4 times higher than rice, 5 times higher than corn and wheat, and almost 10 times higher than potato (Ishizaki, 1997).

The height of each palm varies from 10 until 15 m, while the starch content in each palm depends on several factors such as soil, age, height and planting space. In Peninsular Malaysia, the sago palm is known as *rumbia* like Indonesia, whereas in Sarawak it is called *mulong* (ARC, 1996) or *balau* (Flach, 1997) by the Melanau people and lumbia in the Philippines (Flach, 1977). According to Uhl and Dransfield (1987), sago palm belongs to the order of *Arecales Nakai*, family of *Palmae Jussieu*, subfamily of *Calamoideae* Griffith, tribe of *Calameae* Drude, subtribe *Metroxylinae* Blume and genus *Metroxylon* Rottboel. For hundreds of years, the Melanau people of Sarawak traditionally harvested two main species of sago palm, the thornless (*Metroxylon sagu* Rottbol) and the thorny (*Metroxylon rumphii* Martius) palms for survival (Flach, 1983).

Since then, over 30, 000 ha of sago palm has been cultivated in large-scale plantations provided by the State Government all over Sarawak (Ishizaki, 1997). Presently, the largest sago planting areas are in Oya-Dalat and Mukah with total area of 4,986 ha and 7,928 ha respectively, which involve 15-25 tons/ha of sago starch production (DOA, 2012). It was reported that by 2020, State Government has targeted of having 250, 000 ha of Commercial

Sago Plantation, and the focus of the development is in Mukah division (LCDA, 2013). Thus, continuous development of sago research and systematic cultivation are imperative for this industry to remain competitive against other major and global starch producers.

2.1.2 Sago Industries in Sarawak

Starch is known to be one of the world's most abundant plant products and major source of energy for human consumption. The major sources of starch are corn, potato, tapioca, rice and sago. Sarawak is now the world's largest exporter of sago, exporting about 25,000 to 40,000 tons of sago products annually to Singapore, Taiwan, Japan, Peninsular Malaysia and other countries (Abd-Aziz, 2002). The total area cultivated with sago by districts from 2009-2013 is estimated to be about 54,087 ha with annual production of around 162 tons (DOS, 2015).

The annual export of pure sago starch from Sarawak which fluctuates between 30,000 to 50,000 tons procuring incomes between US$ 3.4 million to US$ 10.8 million (Appendix C). Since 2004, about 40, 000 tonnes of sago starch were exported yearly and had increased to almost 50, 111 tonnes in 2011. In addition, the value of sago starch export was at RM 812/tonnes in 2002 and increased up to RM1, 792/tonnes in 2011, about 55% of increment.

2.1.3 Production and Processing of Sago Starch

Generally, all sago mills in Sarawak utilizes the latest extraction technology whereby the time needed from rasping of the pith to drying and packaging of the starch takes between 30 to 45 minutes (Bujang, 2010). Upon felling of the massive sago palm (9-12 m tall), the trunk is cut into 1 m logs, before the long journey to the sago mill via land (Figure 2a) or river (Figure 2b).

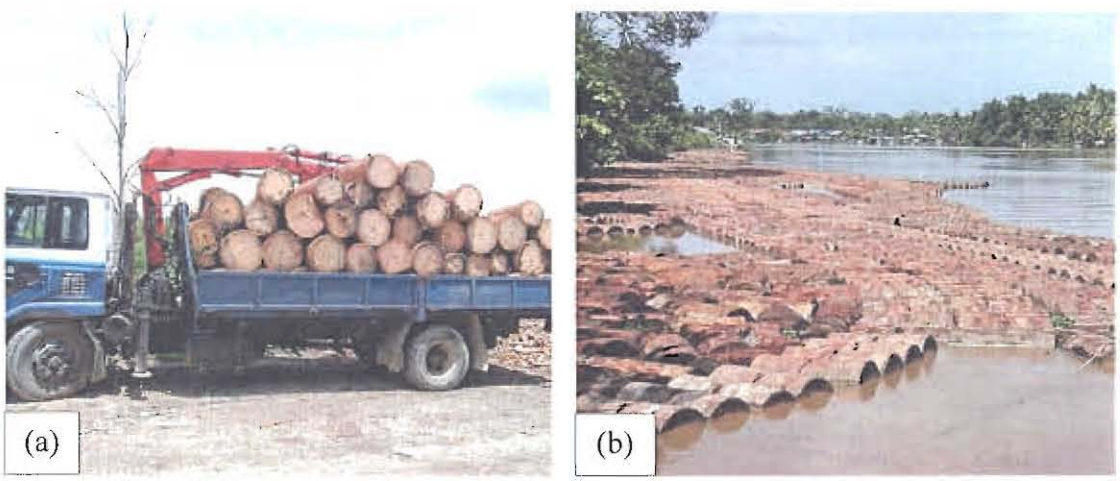


Figure 2: Delivery of sago logs to the local sago mill, using either land transportation (a) or river (b) (Picture courtesy of Ubom Sago Mill, Mukah, Sarawak)



Figure 3: Removal of the sago bark by manual (a) and auto- debarking processes (b) (Picture courtesy of Herdsen Sago Mill, Pusa, Sarawak)

Upon arrival at the mill, the sago pith is obtained by removing the hard bark (about 2-3 cm thick), either by manual (Figure 3a) or automatic (Figure 3b) debarking processes. Although manual process is obviously slower, but the waste on the pith is less due to proper slicing of the bark performed using a heavy machete. Auto-debarking process splits the bark into pieces of uniform size hence easier to store, faster, but leaves higher amount of residual pith under the bark (Bujang, 2010). The debarked pith is macerated using a rotary rasper, followed by addition of filtered water and hammer milling, sieving, settling, washing and

flash drying. Figure 4 represents the overview of starch extraction process in modern local sago mills (Bujang, 2011). The process involves the use of several extractors to extract sago starch from the water upon rasping.

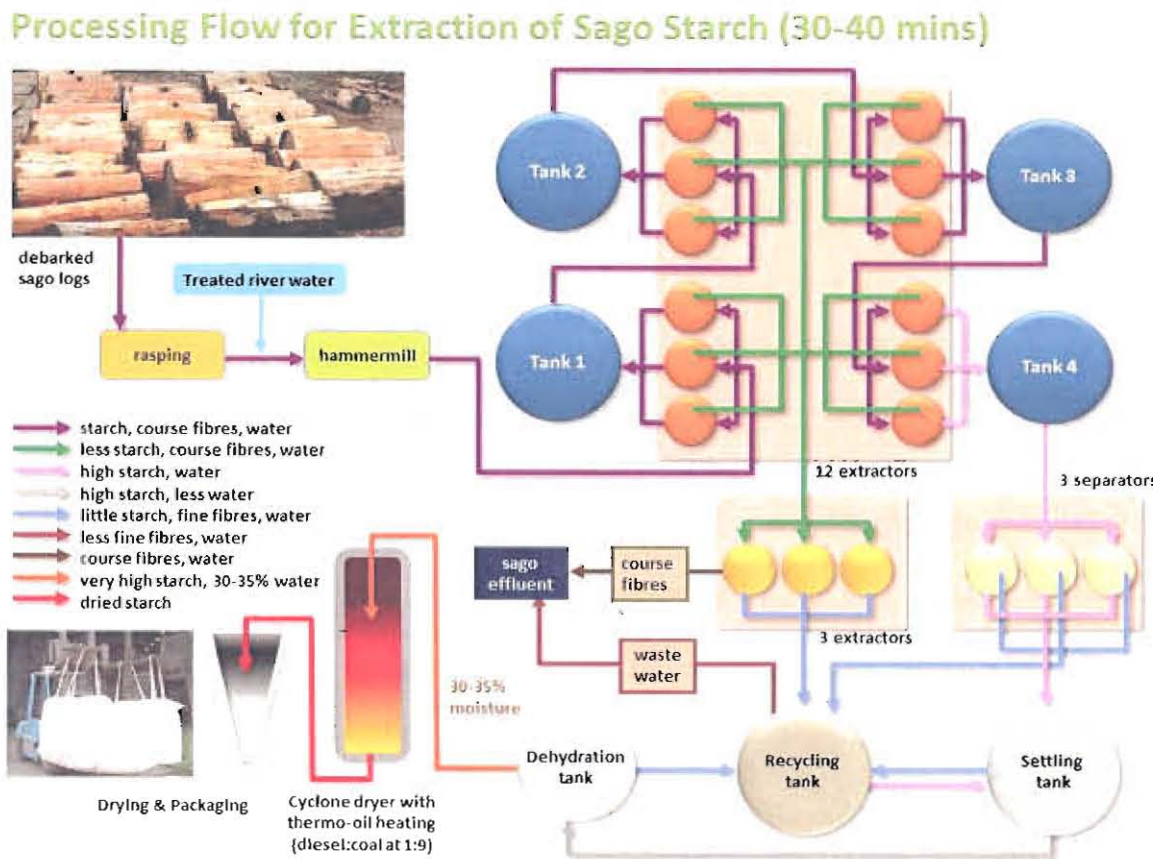


Figure 4: Flow of starch extraction process for a typical sago mill in Sarawak (Bujang, 2011)

Later, a set of separators then separate the sago milk (containing high concentration of starch) from sago effluent, which contains high amount of water and sago fibre. Sago fibre consists mainly of vascular bundles, pulverised to about 1- 2 mm long following the starch extraction step. The separator feeds into the settling tank which may contain between 25 to 40% starch. The waste water is recycled at several times to minimise starch loss into the waste water. Sago starch is deposited at high concentration in the dehydration tank, then dried using a cyclone drier (powered using diesel and co-fired with dried sago bark), packed and ready for delivery.

2.1.4 Sago Palm Product and Residues

Traditionally, all parts of the sago palm have its uses. Other than starch, sago palms are also treasured for the leaves as roofing material and can be woven into hats, baskets and cages of all sizes. The bark is used as firewood, whereas the sticky sap from the frond or rachis is used in making gums and resins, while the palm heart is used in cooking. The sago worms (larvae of *Rhynchophorus schach*) are highly sources as delicacies (Abd-Aziz, 2002). The current trend of utilizing agricultural wastes, i.e. sugarcane baggase, empty fruit bunch and kernel cake from oil palm, paddy or cocoa husks and sago wastes has gained much interests in converting these wastes into value-added products through extensive research. Reports on previous works on the utilization of sago starch and sago wastes (sago fibre, sago bark and sago effluent) are described in Table 1.

Table 1: Reports on the utilisation of sago products

Sago Palm Part	Usage/Utilization	References
Refined sago starch	1. Ingredients of noodles, vermicelli, kuey-teow, biscuits, sago pearls and other food sources. 2. Industrially used in food products such as monosodium glutamate, glucose, caramel, fructose, syrups etc.	Abd-Aziz (2002)
Sago fibre	1. Production of fermentable sugars or reducing sugars. 2. Good substrate for solid state fermentation (as carbon source). 3. Animal feed, bio-composting material, etc.	Abd-Aziz (2002) Awg-Adeni *et al.* (2013) Kumoro *et al.* (2008) Shahrim *et al.* (2008) Yahya *et al.* (2011) Janggu and Bujang (2009)
Sago rachis/fronds	1. Production of fermentable sugars (cellobiose). 2. Used in pulp and paper industries.	Ahmad and Bujang (2014)

Refined sago starch is used mainly as food in the making of noodles and food products such as monosodium glutamate and sugars. Sago fibre has been used for production of fermentable sugars and animal feed, while the sago fronds have been shown to produce cellobiose.

2.1.4.1 Sago Wastes

Many years ago, enhancement of the sago starch industry as a major commercial commodity is delayed by its low production volume due to lack of modern farming strategies and harvesting methods employed by sago mills which ends up with inefficient sago starch extraction process (Karim *et al.*, 2008). Current development in machineries has greatly improved for sago mills in Sarawak. However, during the processing of sago starch, vast amount of untreated wastewater is discharged from sago mills directly into nearby rivers (Figure 5). This affect the quality of river water in the immediate vicinity of sago mills if the disposal method is not modified, or the disposed material is not utilised to reduce pollution.



Figure 5: Release of untreated sago effluent containing starch into a river (photo credit: to K. B. Bujang)

2.1.4.2 Sago Bark

Sago bark (Figure 6) is one of the solid residues from debarking of the log to expose the soft pith for rasping ion starch extraction. Usually, the hard bark is stacked, dried and burned in the boiler together with coal to provide heat for the cyclone dryer. It is also a good source for firewood. Apart from these, bark is used as flooring material in the mill, while locals used it to construct ceilings, fences and wall materials. Bujang *et al.* (1996) estimated that the bark constitutes about 20 % of the weight of each sago log, which averaged at about 130 kg/log. For a medium size sago mill that consumes about 600 logs/day, debarking of these logs to produce starch generates about 15 tons of sago bark/day (Bujang *et al.*, 1996).



Figure 6: Stacks of dried sago barks at a sago mill in Mukah, Sarawak

Recent studies showed that sago bark can be processed with bio-composite material to produce sago plywood, building materials and particleboards (Ruhendi, 2000). Commonly, the shredded form of sago bark can be rebounded with resin and biocompound to produce wall tiles, flower pot and others decoration products (Rahman, 2005). Lignin (25.9%) is the major component of the sago bark and is strongly merged with the hemicelluloses (22.3%) and celluloses (40.8%) in the cell walls of sago pith (Ethaib *et al.*, 2016).

2.1.4.3 Sago Fibre

Sago fibre is the small solids (1-2 mm long) consisting of fibres and vascular bundles separated by filtration from the sago effluent (Figure 7) in sago processing mill. It is a highly potential source of raw material to produce sugar generated by sago industry due to its residual starch attached to the fibres. This solid waste has received considerable attention because it is locally abundant and easy to process as raw materials for production of fermentable sugars and various components (Janggu and Bujang, 2009; Awg-Adeni *et al.*, 2013; Jenol *et al.*, 2014). Recently, it was shown that sago fibre contains significant amounts of residual starch (58%), cellulose (23%), hemicellulose (9.2%) and lignin (3.9%), (Linggang *et al.*, 2012). It was reported earlier that at a daily production of 12 tons of sago starch, a sago mill produces 238 tons of wastewater, and at 3% DM contains more than 7 tons of fibre, all from about 600 logs is released into the river (Bujang *et al.*, 1996).



Figure 7: Sago fibre obtained upon crude filtration of sago effluent (photo credit: to K. B. Bujang)

Hence, the idea is emphasizing on utilising sago fibre being produced daily, convert it into sugar and maximise the use of hydrolysed sago fibre in the fermentation process. Eventually,

this step will minimise further disposal of sago fibre into our rivers which concomitantly will reduce the polluting effects from our sago industries. Other reports on the exploitation of sago fibre are for animal feed, composting materials (Bujang, 1997), confectioner's syrup, particleboard manufacture, and as raw material for local microbes to produce enzymes and reducing sugars have been described elsewhere (Apun *et al.*, 2000; Phang *et al.*, 2000; Rifat *et al.*, 2003; Shahrim *et al.*, 2008).

2.2 Bioethanol

Bioethanol is chemically known as ethyl alcohol (C_2H_5OH), made through fermentation process of plants sugars (i.e. sucrose, glucose, etc.). It is commonly produced from corn, sugarcane and wheat, and the feedstock for biodiesel includes palm oil and its derivatives, alga, soybean, jatropha and rapeseed oil (McIvor and Evans, 2007). Production of bioethanol involves the use of microbe and hence is a fully microbiological process through fermentation. Almost all bioethanol is produced from grain or sugarcane (Mussatto *et al.*, 2010). It is a colourless liquid, which is low in toxicity, environmental friendly and may cause an acute pollution if spilt.

Since 1970s, Brazil and the United States have initiated mass production of bioethanol, derived from starchy and sugary source; corn and sugarcane, respectively. However, the present interests of large-scale bioethanol industries production derived from lignocellulosic wastes material. More cellulosic ethanol will ultimately be produced from varieties of feed stocks, but more fundamental reductions in the cost production will be required prior to make cellulosic ethanol to be commercially viable (Gnansounou and Dauriat, 2005). Hence, by producing bioethanol, some developing countries can minimize the significant amount of oil imported or can replace their existing fuels. Besides, it helps in local air quality advantages

and waste reduction, enhanced vehicle performance, improved rural economic development and protection of ecosystems and soils (Slingerland and Geuns, 2005). The most common usage of bioethanol is to power automobiles through mixed up with petrol at appropriate amount. Ethanol can also be mixed with gasoline in any concentration up to pure ethanol (E100). Anhydrous ethanol, which is at most 1% water, can be mixed with gasoline in varying quantities to minimize the consumption of petroleum fuels and in attempts reducing air pollution and the most interesting fact is the usage of bioethanol are generally CO_2 neutral. This can be achieved because in the growing phase of the plant sources, CO_2 is absorbed by the plant and oxygen is released at the same volume that CO_2 is generated in the fuel combustion. Production of ethanol from corn is shown in Figure 8.

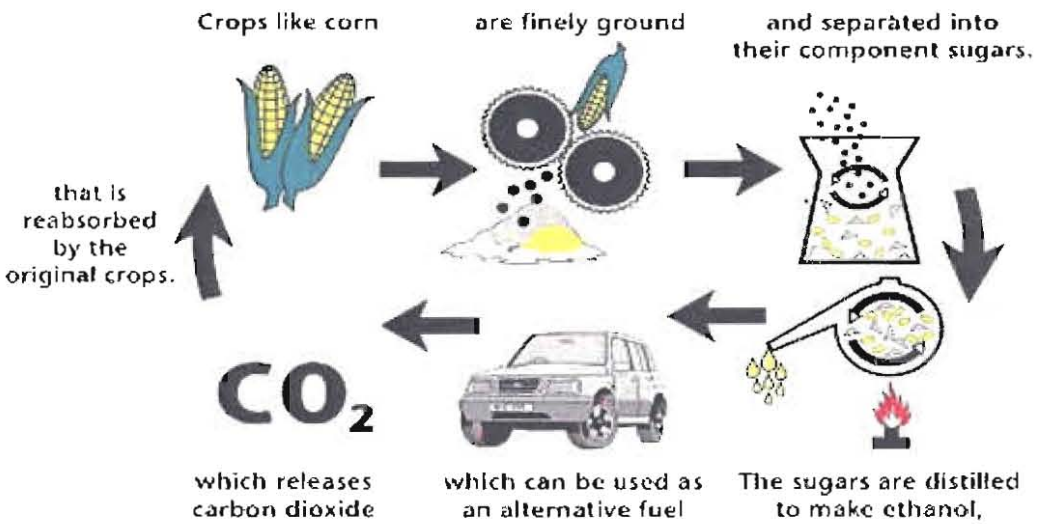


Figure 8: Production of ethanol as biofuel from corn (Sources: Energy Information Administration, Annual Energy Review 2005)

Wyman (1996) reported that the usage of petrol for transportation sectors accounts for one third of the greenhouse gas emissions. This fact is supported by Demirbas (2007) where worldwide energy consumption has increased 17-fold in the last century where the emissions of CO_2, SO2 and NO_x primarily contribute to atmospheric pollution.

2.2.1 Bioethanol Industries and Global Market

Bioethanol is produced and used in several countries around the world, for example, in United States (USA), Brazil, Canada, Japan, India and Europe (Mussatto *et al.*, 2010). Current interest in bioethanol grows rapidly worldwide due to the expected shortage of fossil fuels in the next 20 years or so. Hence, bioethanol generation from lignocellulosic biomass has been promoted as a promising alternative to replace fossil fuels due to many advantages. The industry would able to increase feedstock availability and reduce feedstock costs but requires the advancement and innovative processes of pilot scale production. Figure 9 shows the different processes for bioethanol production from starch and lignocellulosic biomass (Chiaramonti, 2007).

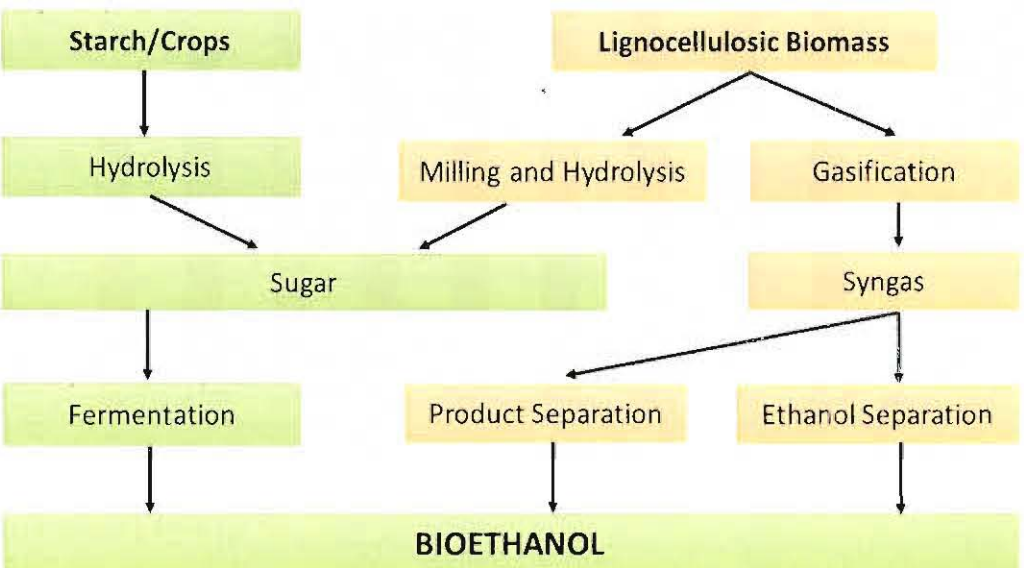


Figure 9: Main pathways of bioethanol production (Chiaramonti, 2007)

The demand on green biofuel which is ethanol has increased over the years due to the increment of environmental issues such as global warming, petroleum crude oil shortage, the very high cost of oil and political instability of several crude oil producing countries (Saifuddin *et al.*, 2011). It was expected in the year of 2050, the global oil production started

to decline from 25 billion barrels to 5 billion barrels (Campbell and Laherree, 1998). Hence, it is necessary and very promising to utilize the potential of renewable sources such as starch and lignocellulosic feed stocks to produce liquid biofuels such as bioethanol and biodiesel when the oil prices show continual fluctuation. Ethanol is the world largest biofuel and is expected to remain so. Over the years, the production of ethanol increased dramatically even from 2007 to 2015. Figure 10 shows the global ethanol production by countries, mainly for fuel purposes, which has risen over the past several years.

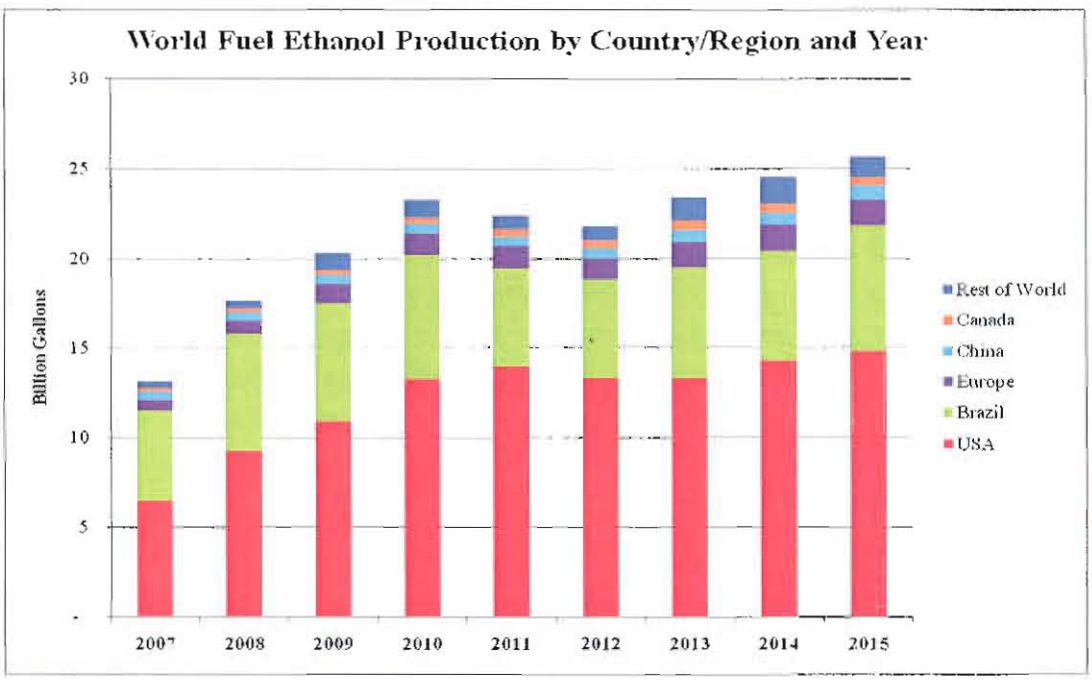


Figure 10: World Fuel Ethanol Production from 2007 to 2015. (Retrieved from Renewable Fuel Association, 2016)

Globally, United States (USA) and Brazil are the current leading nation compared to Asian countries serves as the main suppliers of ethanol which altogether accounts for 14% of the world's production (Carere *et al.*, 2008). From Figure 10, in 2015, the bioethanol market is expected to reach about 100 x 10^9 litres, where the United States use corn as their feedstock for producing 35.5 x 10^9 litres of ethanol. Brazil uses sugarcane to produce 30 x 10^9 litres.

Recently, China is also categorized as one of the largest producer of ethanol in the world (Ivanova *et al.*, 2011).

2.2.2 Applications of Bioethanol

Bioethanol is widely used as liquid biofuel in automotive transportation sectors. However, this energy saving fuel is also being used for many other sectors such as in chemical/detergent industry, alcoholic beverages and medical. Table 2 shows several applications of bioethanol in different sectors.

Table 2: Applications of bioethanol in various sectors

Sectors	Application	References
Chemical industry	Ethanol is categorized as multipurpose product necessary for the powerful chemical industry. It is used to produce list of industrial chemical products and highly performing solvent/detergent for agro industries preparations.	Chandel *et al.* (2007)
Alcoholic Beverages	Ethanol is used to produce various types of liquor such as beer and wine.	Demirbas (2007)
Transportation	Bioethanol used as petrol additive that can be used as a 5% blend with petrol under EU quality standard EN228.	Demirbas (2008)
Medical	Bioethanol had undergone bioconversion by yeast to produce probiotics and bio-therapeutic agents.	Alam *et al.* (2007)

2.2.3 Substrates for Production of Bioethanol

Recently, fuel ethanol has been commercially produced using sugary or starchy agricultural crop (Thomas and Kwong, 2001). Moreover, technology to produce ethanol from lignocellulosic biomass feed stocks have been developed and tested in various scale (Drapcho *et al.*, 2008).

2.2.3.1 Starch-Based Bioethanol

Varieties source of starchy materials have been utilized successfully for the bioethanol production (Jagatee *et al.*, 2015). Cassava starch has great potential to be the substrate of bioethanol production commercially due to its high starch content (70-85%), fast-growing crop and available mainly in tropical countries (Balat *et al.*, 2008; Perves *et al.*, 2014). Prior to standard hydrolysis process, cassava starch requires gelatinization steps to produce sugar. Then, sugar obtained was further used in bioethanol fermentation by using *Saccharomyces cerevisiae* (Zamora *et al.*, 2010). In the study performed by Puligundla *et al.* (2011), production of ethanol via very high gravity of liquefied cassava mashes using flocculating yeast *Saccharomyces bayanus* has demonstrated that effective bioethanol production with final ethanol concentrations of 101.8 g/L and 94.6 g/L and fermentation efficiencies of 75.8% and 57.8%, respectively.

Sweet potato can also be used as substrate in the alcoholic fermentation since it contains higher starch yield, approximately 85% of total world output of this raw material (Zhang and Feng, 2010; Zhang *et al.*, 2011). The dry matter content in sweet potato ranges from 21-30% which comprised of 80% starch. Commonly, industrial sweet potatoes are bred to increase its starchy content, which ultimately reduced its attractiveness as a food crop when compared to other conventional food cultivars. As been reported, some industrial potatoes can produce

up to 4500-6500 L/ha compared to 2800-3800 L/ha for corn (Ziska *et al.*, 2009; Duvernay *et al.*, 2013).

In recent years, Lareo *et al.* (2013) conclude that by using fresh and dry flour of sweet potatoes can generate up to 38-45 g/L of ethanol concentration within 16 h of fermentation time with 100% sugar conversion. It was estimated around 4.9 billion bushels of corn was exploited for bioethanol industry in the USA from which approximately 13.2 billion gallons of ethanol can be produced (Chen *et al.*, 2013). Starch can be obtained from corn through the milling process, further enzymatically hydrolysed to obtain glucose syrup, and then fermented into an alcohol. A comparative analyses of bioethanol production derived from various starchy crops has been summarized in Table 3.

Table 3: Comparative analysis of various starchy crops involved in the current and future ethanol production

Starchy Crops/Substrates	Ethanol Production (L/ha)	References
Cassava	4711	Drapcho *et al.* (2008)
Sweet potato	4800	Lareo *et al.* (2013)
Potato	1800	Osuji *et al.* (2010)
Corn	2430	Kraatz (2008)

2.2.3.2 Cellulose-Based Bioethanol

Naturally, lignocellulosic biomass is made up of cellulose, hemicellulose and lignin. Cellulose exists as a linear, crystalline homopolymer with repeating units of glucose holds together by beta-glycosidic linkages. The structure is firm and required harsh pre-treatment in order to break it down. Hemicellulose is a short, linear and highly branched chains of sugars. Differ from cellulose, it is a heteropolymer of D-xylose, D-glucose, D-galactose, D-mannose and L-arabinose. Commonly, cellulose-based feed stocks derived from cellulosic

biomass such as agricultural residues, wood residues, industrial and other wastes are widely used to produce bioethanol in commercial industries. These feed stocks are seen to be more challenging compared to starch feedstock in term of releasing its sugars for conversion of ethanol due to its complex structure of raw material. Wheat straw, corn stalk and cobs, and alfalfa fibre are the examples of some crop residues; rich in lignocellulosic which have been exploited for ethanol fermentation. Table 4 represents the advantages of some agricultural residues for ethanol production (Prasad *et al.*, 2007).

Table 4: Advantages of some agricultural residues for ethanol production

Agricultural Residues	Advantages
Corn Fibre	Enriched in hemicellulose, produced from corn hull during the wet milling of the corn.
Rice Straw	Easily available agricultural wastes in bulk, contained about 40% cellulose, 18% hemicellulose and 5.5% lignin.
Alfalfa Fibre	Comprised of cellulose, hemicellulose, lignin, pectin and protein. High potential in the production of juice derived co-protein.

2.2.3.3 Fermentable Sugars from Sago Industries

Technically, any plant materials able to produce biofuel if the starch and cellulose are readily available and convertible into fermentable sugars (Bujang, 2008). Among all the starchy plants of the world, the sago palm has been providing our countries with vast amount of sago starch as a food source and so forth. Sago starch, easy to gelatinize can be further enzymatically hydrolysed by using α-amylase and amyloglucosidase enzyme, which subsequently produce glucose and others fermentable sugars. Sugars from hydrolysis of starch such as glucose syrup, maltodextrin, corn syrup, and high fructose syrup have been

used in food industry, brewing, ethanol and pharmaceutical industries (Subashini *et al.*, 2011; Soto *et al.*, 2012). Sunaryanto *et al.* (2013) also reported that sago starch was completely utilized as feed stocks in ethanol fermentation industries and high-fructose syrup industries.

Previously, in the Biochemistry Laboratory, at Faculty Resource of Science and Technology, UNIMAS, ethanol from sago starch (raw or hydrolysed) sugar has been reported to be feasible as substrates for production of bioethanol. Study conducted by Bujang et al. (2000a) showed about 40 mL/hr of ethanol can be generated from each kilogram (kg) of sago starch, based on Ishizaki process, a high-speed process developed in pilot-plant bioethanol project in UNIMAS. Moreover, the study was continued with different system; continuous mode of ethanol fermentation using various supplementation of nitrogen sources (yeast extract 5 g/L, Mieki 2% v/v and corn- steep liquor 4% v/v) on hydrolysed sago starch (HSS) by using *S. cereviceae* CSI-1 (Niyong and Bujang, 2009). Based on the findings, it is proven that sago starch generated high amount of bioethanol, whether in lab scale or even pilot scale.

However, due to high demand of sago starch consumption, the upward trend in sago production will totally increase the amount of wastes generated from this sago industry; including sago fibre, sago bark and sago wastewater. Sago fibre, which is a free biomass, readily available and abundant, still consists high amount of trapped starch (50-60%) and exploited by most researchers for sugar production. Previously in UNIMAS, studies on sago fibre was initiated in the year 2008 and until now. Research studies utilising the starchy and lignocellulosic material of sago fibre into fermentable sugars have been successfully conducted and reported (Janggu and Bujang, 2009; Awg-Adeni *et al.*, 2010; Linggang *et al.*, 2012; Vincent *et al.*, 2015).

Preliminary studies done by Awg- Adeni *et al.* (2013) indicated that residual starch of sago fibre was enzymatically hydrolysed into sugars with total 80-90% recovery through recycling method. Sugars obtained from the process mainly contains glucose and considered as a complex fermentation media in producing bioethanol by *S. cerevisiae*. From the study, ethanol concentration was at 40.30 g/L, which equivalent to 93.29% of fermentation efficiency after 16 hours of fermentation period (Awg-Adeni *et al.*, 2013). Moreover, some basic studies on nutrient supplementation have been conducted with hydrolysate of sago fibre as fermentation media for producing bioethanol.

2.2.4 Utilization of Microorganisms in Ethanol Fermentation

Varieties species and strains of yeast, bacteria and moulds have been reported as the fermentative microorganisms that converting sugar into ethanol. The most common and commercially worldwide microorganisms for ethanol production are the correlated species and strains of *Saccharomyces cerevisiae* (Lin and Tanaka, 2006; Chandel *et al.*, 2007).

2.2.4.1 Yeast

Yeasts are unicellular fungi which commonly found in natural environments (Curry, 2009), however some species would become multicellular fungi by formation of budding cells, known as false hyphae. It can be classified based on their ascospore, colonies and its physiological characteristics. Budding is the common mode of yeast vegetative growth (Legras *et al.*, 2007). Particularly, yeasts members of the genera *Saccharomyces, Schizosaccharomyces, Candida, Pashysolan, Pichia* and *Kluyveromyces* are the primary interest towards industrial fermentation operations which generating bioethanol.

Yeasts structure comprised of single cells which are smaller than animal and plant cells but larger than bacteria. Outside layer of the yeast cell covered by cell wall and inside of the cell contains cytoplasm having a nucleus, mitochondria and ribosomes. The cell wall is made up of a two-layered structure, the inner layer is made up of β-1-3 glucane linked covalently to chitin and it is responsible for yeast cells stability, whereas the outer layer consists of densely packed glycosylated mannoproteins which reduce the permeability. Although the cell wall provide stabilization to the cell, the cell membrane which comprise of phospholipids and proteins forms the main wall for substrate exchange in and out of the cell (Figure 11). The most highlighted part of glycogen granules, which build up at the end of fermentation; serve as an energy reservoir during storage and starting energy for new cell cycles to begin (Tenge, 2009).

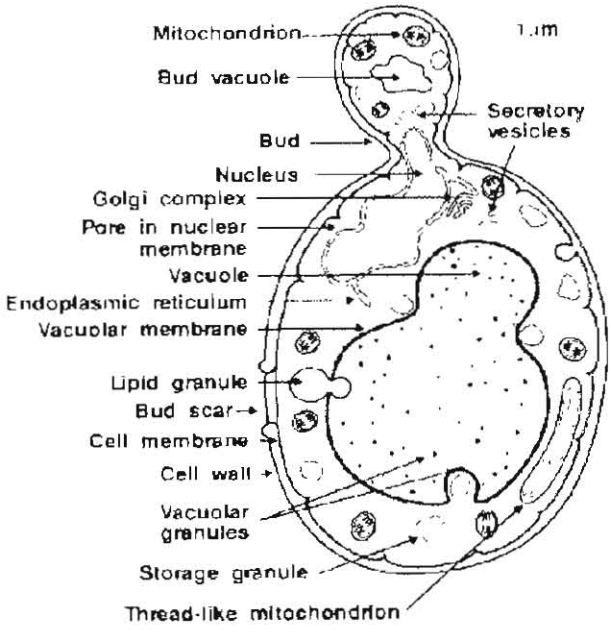


Figure 11: Cross section of a yeast cell
(Retrieved from http://www.bakeinfo.co.nz/Facts/Bread-making/Bread-ingredients/Yeast, February 16, 2016)

2.2.4.1.1 Metabolism in Yeast

Yeasts can utilize broad types of sugars and carbohydrates. It only requires a reduced carbon source, various minerals and adequate supply of nitrogen and vitamins. Yeast cell metabolism (i.e., by aerobic respiration or by fermentation) is based on the concentration of sugar to which yeast cell is exposed. For instance, when the sugar concentration is high, the yeast cell is switched into the fermentative mode, and pyruvate is metabolized via acetaldehyde to ethanol, yielding 2 molecules of ATP per sugar molecule. However, at low sugar concentrations, the pyruvate shunts into acetyl-coA and the respiratory chain, which yields 32 molecules of ATP per sugar molecules (as shown in Figure 12).

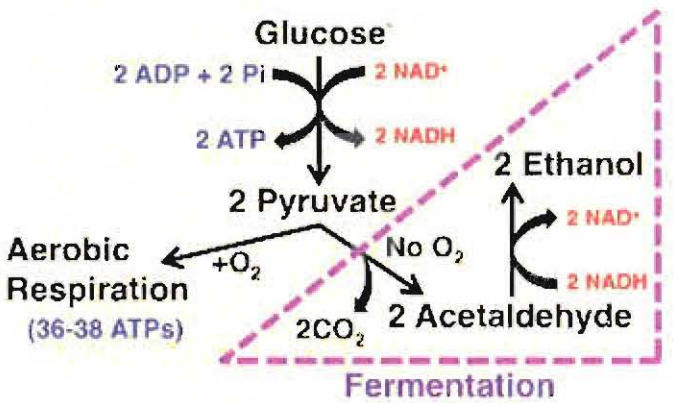


Figure 12: Metabolic pathway of ethanol fermentation in *S. cerevisiae* (Source: Madigan *et al.*, 2000)

This phenomenon is called as the Crabtree effect. The yeast cell cannot produce many ATP molecules when sugar is limited. Hence, in any fermentation industries, employing high sugar concentration as substrate would generate high alcohol yield as the product. In the production of baker's yeast, where the requirement is high cell yield, the sugar

concentrations is always kept low, but sugar is continuously passed into the fermenter. Commercial baker's yeast under anaerobic conditions metabolizes glucose to ethanol by Embden-Meyerhorf-Parnas (EMP) pathway, through one molecule of glucose is metabolized, and two molecules of pyruvate are produced (Lin and Tanaka, 2006). Production of ethanol from glucose can also be expressed by the following equation:

$$C_6H_{12}O_6 + 2\ Pi + 2\ ADP \longrightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP + 2\ H_2O$$

During anaerobic condition, pyruvate is transformed into ethanol and carbon dioxide, CO_2 is released. Theoretically, a mass basis of glucose metabolized yield 0.511 ethanol and 0.489 CO_2. Hence, two ATPs were produced during the glycolysis process are used to produce yeast cells which involved a variety of energy-requiring bioreactions. Apart from cell biomass and gas, a few by-products were also generated such as glycerol (estimated 5%) and other organic acid. Glycerol is a common fermentation by-product. It has a role in protecting cell against high osmotic pressure and heat shock (Russell, 2003).

2.2.4.1.2 Yeasts in Fermented Foods and Beverages

Currently, yeasts are widely utilized for various types of industries, including fermented food, and beverages specifically in brewing or fermentation industries. Across the world, these industries have important roles in the production of food as well as food safety. Yeasts, alone or in stable mixed with fungi or bacteria has substantial impacts on quality of food with various parameters such as texture, odour, taste, nutritional values, shelf-life and so forth.

2.2.4.2 Commercial Baker's Yeast

Commercial baker's yeast is one of the well-known ethanol producers. It can produce high concentration of ethanol at a very suitable nutrition condition and environment (Bafrncova *et al.*, 1999; Lin and Tanaka, 2006). Other species related to baker's yeast are *Saccharomyces oviformis*, *Saccharomyces italicus*, *Saccharomyces capensis* and *Saccharomyces uvarum var. melibiosus* (NCBI, 2016). Previously, baker's yeast has been widely used to ferment sugars of wheat, rice, corn and barley which produce alcoholic beverages and bakery (Tsuyoshi *et al.*, 2005). Baker's yeast has short germination time and can be easily culture in large scale processes (Shafagat *et al.*, 2010). Study done by Laopaiboon *et al.* (2009) observed that ethanol production was improved when 3.0 g/L of yeast extract (120.68 ± 0.54 g/L) was added to sweet sorghum juice under very high gravity (VHG) fermentations by *S. cerevisiae*.

Baker's yeast, like all microorganisms, goes through different stages of growth (lag phase, logarithmic phase, stationary phase, and death phase). The growth of yeast and alcohol production are coupled phenomenon and usually growing actively yeast can produce ethanol thirty-three times faster compared to the stationary phase. Thus, if yeast cells can be kept in its active phase for longer period, the overall fermentation time could be decreased, and the amount of sugar consumed to ethanol would be maximized. Apart from that, commercial baker's yeast is excellent in terms of ethanol productivity and highly tolerance towards alcohol compared to the other strains (Ahring, 2007). It can grow at low pH values to avoid bacterial contaminations. However, ethanol fermentation with other types of yeast; active dry yeast, liquid yeasts and compressed yeast can also be employed. Normally, active dry yeast is categorised by a longer lag phase because of the rehydration and acclimatization to compensate with the drying process stresses, whereas yeasts in liquid form can be used faster

and possess very high vitality, which resulted in superior fermentation process. Commonly, baker's yeast was packed as slurry (yeast cream), active dry yeast and compressed yeast in various fermentation industries. Table 5 below represents several examples of ethanol producer with different raw materials that can be utilized as substrates. Other yeasts that can convert sugar into bioethanol are *Candida shehatae*, *Pichia stipitis* and *Pashysolan tannophilus* (Chandel *et al.*, 2007).

Table 5: Various substrates with different fermentation condition that use yeast for bioethanol production

Raw Material/Substrate	Fermentation Mode	Microorganism	Ethanol (g/L)	References
Corn/Potato Starch	Batch	*Candida tropicalis*	56	Jamai *et al.* (2007)
Corn Meal	Batch	*S. cerevisiae*	77	Majovic *et al.* (2012)
Municipal solid waste (MSW)	Batch	*S. cerevisiae*	0.248	Mtui and Nakamura (2005)
Sago Starch	Batch	*Z. mobilis* MTCC 92	50	Bujang *et al.* (2000b)
Sweet Sorghum	Batch	*Fusarium oxysporum* and *S. cerevisiae*	35-49	Mamma *et al.* (1996)
Soybean Molasses	Batch	*S. cerevisiae*	43.00	Siquiera *et al.* (2008)
Sago fibre	Batch	*S. cerevisiae* (Commercial baker's yeast)	40.30	Awg-Adeni *et al.* (2013)
Salix (fast-growing willow)	SSF	*S. cerevisiae*	7.90	Sassner *et al.* (2008)

2.2.4.3 Bacteria

A great number of bacteria are gifted in generating ethanol by fermentation process. These bacteria that can produce ethanol as the major product (i.e. produce a minimum of 1 mol of ethanol per mol of glucose utilized) includes *Clostridium sporogenes*, *C. indolis*, *C. sphenoides*, *C. sordelli*, *Kliebsiella oxytoca*, *Z. mobilis* (Lin and Tanaka, 2006), *S. litoralis*, *S. stenostrepta and E. coli* strain K011 (Zabed *et al.*, 2014). The bacterium, *E. coli* is a valuable bacterial resource for ethanol production. It is widely used in fermentation process because of its efficiency in transforming wide spectrum of sugars into alcohol, able to flocculate during growth and highly tolerance towards ethanol (Zabed *et al.*, 2014). However, major disadvantages associated with *E. coli* cultures are narrow and neutral pH growth range (6.0-8.0) and less hardy cultures compared to yeast. However, among the other species of bacteria, *Z. mobilis* also stands out for high ethanol yield and productivity because of its unique physiology. It is gram-negative bacteria that pursue several appealing properties as a biocatalyst for ethanol production.

This microbe has a homoethanol fermentation pathway and tolerates up to 120 g/L ethanol. It has higher ethanol yield (5-10% more ethanol per fermented glucose) and has much higher specific ethanol productivity which are about 2.5 times higher than *Saccharomyces* species itself (Sprenger, 1996). *Z. mobilis* catalyzes glucose by Entner-Duodoroff (ED) pathway as opposed to the Embden-Meyerhorf (EM) or glycolytic pathway (Mathews and Wang, 2005) and it is the only microorganisms that can metabolize glucose anaerobically. However, *Z. mobilis* produces less biomass than the yeast *(S. cerevisae)* and more carbon is funnelled to the fermentation products. Inspite of the advantages of *Z. mobilis*, this microbe is not well suited for all the raw materials/biomass conversion because it can only ferments glucose, fructose and sucrose; thus, making it hardly to replace *S. cerevisiae* (Zabed *et al.*, 2014).

Recently, *Z. mobilis* and *E. coli* have been also targeted through metabolic engineered for cellulosic ethanol production. The strain was modified to become alcohol-tolerant strains for fermentation process. Besides, the aim is to obtain a high bioethanol yield using the engineered strains that generate bioethanol with few side products, and metabolize all major types of sugars including glucose, xylose, galactose, mannose and arabinose. Currently, engineered microorganisms was introduced and used by researchers in converting lignocellulosic biomass into ethanol.

2.2.5 Factors Influencing Fermentation Process

Commonly, in any fermentation process, there are several factors which can affects the overall process including temperature, pH, nature and composition of the medium, dissolved oxygen, dissolved carbon dioxide, operational system (e.g. batch, fed-batch, continuous), feeding with precursors, mixing and shears rates in the fermenter. Variations on these factors could affect: rate of fermentation; the product spectrum and yield; the organoleptic properties of the product (taste, smell, texture and appearance); the toxins generations; nutritional quality; and other physico-chemical properties. Moreover, the formulation of fermentation medium also affects the ethanol yield, rate and product profile. Commonly the fermentation medium must contain necessary amounts of carbon, nitrogen sources, trace elements and micronutrients (e.g. vitamins). Specific types of carbon and nitrogen sources may be needed and the C: N ratio must to be controlled. Concentration of certain nutrients must also be varied in a specific way during fermentation in order to achieve desired results. Additional factors, such as availability, cost of substrates, and system variability also affects the choice of medium fermentation.

2.2.5.1 Availability and Costs of Substrates

Regardless of the cost and availability of raw materials, commonly, sugary substrates require less pre-treatment cost compared to the starchy or lignocellulosic materials and other urban wastes material which need costly pre-treatment to transform raw materials into fermentable sugars (Sun and Cheng, 2002). However, starchy substrates are the most utilizable nowadays due to its abundance. Ethanol production from raw sago starch and continuous ethanol fermentation system from sago starch sugars are commonly employed (Jong, 2001) as well as starchy lignocellulosics waste from sago palm, which is sago fibre. Those sago fibres; which are free and abundant are more crucial to utilize because it creates environmental pollution and consequently caused deterioration towards the environments (Awg-Adeni *et al.*, 2013).

2.2.5.2 Yeast Growth Requirements

During fermentation process itself, several environmental stresses including caused by changes in pH, temperature, osmotic pressure, nutrient availability and ethanol concentration affects the yeast cell. Normally, it can individually or collectively cause deleteriously impacts on the physiology of the yeast and diminished the performance of fermentation process itself (Walker and Dijck, 2006). Thus, it is crucial to understand the ways by which yeasts responds to the physiochemical environment in the industries. Referring to Russell (2003), the yeast cell require water, carbon source which consists of fermentable sugars as energy source, oxygens or lipids that essentials for membrane biosynthesis, source of nitrogen, growth factors such as vitamins and inorganic ions that are crucial for the cell growth and cell metabolism.

In any ethanol fermentation process, the medium used must be nutritious and consist of a fermentable carbon source such as sago starch sugar or sugarcane and amino acids. During initial stage of fermentation, oxygen, vitamins and minerals are required together with minor growth factors as in the medium formulation part. Usually, the best temperature for most laboratory and industrial yeasts are between 20-30 °C. Specifically, for *S. cerevisiae,* maximum temperature values range from 35 °C and 43 °C, whereas other strains of *S. bayanus* and *S. pastorianus* fail to grow above 35 °C.

2.2.5.3 Carbon Source for Yeast

Naturally, carbon source is needed for energy purposes, as well as growth factors towards microbial growth instead of water. Microbes can easily get their carbon sources, either from organic materials such as carbohydrates and amino acids. However, there are certain microbes that easily obtain carbon source directly from carbon dioxide. In anaerobic environments, yeasts can convert simple sugars, such as glucose into ethanol and carbon dioxide gas. Most commonly sugars used in fermentation such as hexoses (glucose, fructose, galactose and mannose) and disaccharides (maltose or sucrose) as well as compounds with two carbon ethanol and acetate (Rodrigues *et al.*, 2006).

Moreover, sucrose can be consumed faster by the yeast's invertase and the profile reduced very quickly from the substrate. If a very high glucose concentration compared to others sugar is present inside the fermentation tank, one can encounter stuck fermentation due to glucose repression. As a result, the yeast is not able to synthesize the enzymes assimilate with sugars present. Thus, low ethanol yield can be achieved rather than optimal yield.

2.2.5.4 Nitrogen Sources for Yeast

Nitrogen source is required by the yeast during fermentation. It is an essential component for the cell growth especially in manufacturing cellular materials. Without nitrogen, the synthesis of DNA and RNA cannot be performed. Inadequate sources of nitrogen would also cause to a reduction in ethanol yield, low biomass yield, slower rate of fermentation and increase risk of sluggish fermentation (Junior *et al.*, 2012). During fermentation, some of the nitrogenous compound are taken up and consumed directly by the yeast cell, but some are also degraded to release nitrogen and used by the cell.

Previous researches have shown that ammonium ion (Yue *et al.*, 2012), Urea (Pereira *et al.*, 2010; Nofemele *et al.*, 2012), peptone (Laopaiboon *et al.*, 2009), yeast extract (Duhan *et al.*, 2013), corn steep liquor (Niyong and Bujang, 2009) and other nitrogen sources could improve the yeast growth and increase ethanol production. However, not all nitrogen sources contribute to yeast growth equally well (Beltran *et al.*, 2004). Some research did show that yeast extract and peptone give more positive results than urea and ammonium sulfate (Sridee *et al.*, 2011). Yeast extract mostly contained peptides, amino acids and vitamins, by which those components are very helpful in the fermentation because peptides and amino acids are the building blocks of the cell. Ammonium salts stimulate glucose fermentation by lowering induction period and maintaining an optimum carbon to nitrogen (C/N) ratio. According to Wang *et al.* (2012), high yield of glycerol and acetic acid were observed when using ammonium sulfate as the nitrogen supplements. Higher rate of ethanol production and growth rates was achieved when using urea and ammonia as nitrogen source. According to Ananda *et al.* (2011), media supplementation components could contribute about 38% of total production costs; hence significantly give impacts on the economics of industrial fermentation sector. Due to the shortage of the raw material which result in 10% increase of

the yeast extract price. Thus, different raw materials require different types of nitrogen sources, because the assimilation of nitrogen inside the fermentation media is crucial to perform an efficient and successful fermentation process.

2.2.5.5 Metal Ionic Compound for Yeast

Supplementation of metal inducers at certain concentrations instead of nitrogen and phosphorus are also required by the yeast cells in ethanol production (Udeh *et al.*, 2013). Magnesium (Mg^{2+}), calcium (Ca^{2+}), and zinc (Zn^{2+}) were claimed to encourage sugar conversion rate as well as metabolic changes in yeast biomass growth (Aleksander *et al.*, 2009). Usually, very low amount of trace elements was added into fermentation media (Cao and Liu, 2013) due to several factors which can affect the glycolysis pathway. Magnesium supplementation was reported to maintain the cell membranes, which contributes much in the alcoholic concentration itself (Somda *et al.*, 2011). Calcium supplementation is mainly for the growth of yeast cells as it was involved in membrane structure and for yeast flocculation (Russell, 2003; Walker *et al.*, 2006). Supplementing zinc in the fermentation medium assist to stimulate cell growth and cell metabolism (Zhao *et al.*, 2009) and without zinc, the fermentation process become slow (Russell, 2003). The addition of zinc not only promote the specific cell growth rate, but also assisting fermentation process as activator of the terminal alcohologenic Zn-metalloenzyme ethanol dehydrogenase (Walker *et al.*, 2006).

2.2.6 Fermentation Technology

Nowadays, fermentation technologies are widely exploited by researchers in many environmental and economical industrial sectors. These technologies portray great advantages of low cost substrates, high product specificity and low energy consumption rather than chemical synthesis process. Technically, industrial fermentations can be carried

out either in batch, fed-batch, continuous or semi continuous modes. In the continuous processes, sterile medium is fed continuously into a fermenter and the fermented product is continuously withdrawn, so the fermentation volume remains unchanged. Typically, these systems started with batch cultures and feeding begins after the microbial population has reached certain concentration. In this system, there is no need for a separate inoculum vessel, except at the initial start-up. However, there is a high risk of microbial contamination and mutation due to long cultivation period and periodic handling. Furthermore, this system requires larger reactor volumes, thus slightly higher investment costs are needed.

Differ from continuous operations, fed-batch mode started with a small amount of yeast cultures and substrate in fermenter, then, a feeding medium was added, stepwise or continuously to the fermenter, when most of the initially added substrate has been consumed. These operations could increase the total substrate content in the fermenter, while maintaining a low substrate concentration during fermentation to reduce negative effects on yeast osmotic pressure (Phukoetphim *et al.*, 2017). The main advantages of this fed-batch system include reduction of substrate inhibition, higher productivity, shortened fermentation time and reduction of toxic effects on the medium components. Despite all the systems, batch mode possesses more advantages particularly in ethanol fermentation. Currently, most of the ethanol produced is done by batch operation since the investments costs are low, do not require much control and can be done with unskilled labour. In batch fermentation, the substrate and yeast (starter culture) are inoculated into the fermenter without removal of media during fermentation and the products are harvested at the end of the fermentation process. Batch fermentation typically extends over 3-5 days, but some traditional food fermentations may last months. The volume of fermenting broth increases with each addition of the medium, and the fermenter is harvested after the batch time. Furthermore, complete

sterilization and management of feed stocks are easier in batch mode compared to other processes (Najafpour *et al.*, 2004).

In this context, the economy of the fermentation process should enhance the implementation of bioethanol which can be sourced from sago fibre. Sago fibre is locally abundant wastes and can be processed easily as an alternative substrate for the biofuel industry compared to other feedstocks. This practice will concomitantly conserve our environment from pollution. Moreover, supplementation of sago fibre hydrolysate with less expensive nitrogen sources such as ammonium sulfate, urea and none of metal ionic components would minimize the overall costing of bioethanol production. High demand of sago starch from the ASEAN countries would also concomitantly increase the starch production thus releasing huge amount of waste at the processing sago mill. Sago fibre which contains mostly residual starch (59-60%) serve as alternative substrate for generating glucose, which then be used as carbon source for producing eco-friendly bioethanol through fermentation process by baker's yeast. Even though, low amount of ethanol was commonly generated due to low concentration of glucose feed at initial stage of the fermentation processes. It should be noted that any agricultural wastes material has complex and rigid physical appearance which serves as obstacles in transforming it into glucose. Hence, it is very important to conduct an efficient enzymatic hydrolysis, which simultaneously producing glucose in the form of economical fermentation medium for the baker's yeast to consume the carbon source. A successful and efficient ethanol fermentation system must consider all the key factors and lessen by-products formation together with high ethanol yield conversion and productivity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Sago Fibre

Sago fibre samples used as the raw material for glucose production were obtained from Herdsen Sago Mill Sdn. Bhd near Pusa and Ubom Sago Mill near Mukah, Sarawak in dry condition (Figures 13a and 13b). The samples were packed into porous plastic bags prior to delivery to UNIMAS where it is kept in suitable containers at room temperature before further analyses.

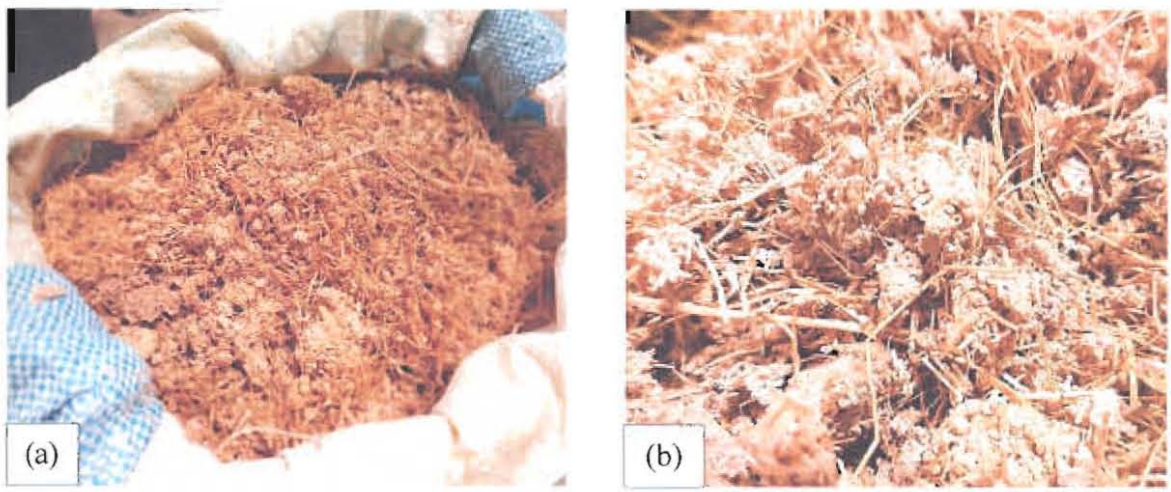


Figure 13: Sago fibre obtained from Ubom Sago Mill, Mukah. Dried sago fibre in porous plastic bag (a) and close-up photo of dried sago fibre (b)

3.1.2 Commercial Glucose (CG)

Commercial glucose (Sigma-Aldrich Company, USA) act as a positive control of carbon source for production of bioethanol by commercial baker's yeast (CBY) was used throughout this study.

3.1.4 Commercial Baker's Yeast (CBY)

A commercial brand of baker's yeast (Figure 14) is the chosen source for *Saccharomyces cerevisiae*. The yeast is in the form of active dried yeast pellets, supplied by Mauripan AB Mauri Malaysia Sdn. Bhd. The dried yeast was activated in Yeast Malt Broth (YMB) medium and maintained in glycerol stock at 0 °C.



Figure 14: Commercial baker's yeast (Mauripan)

3.1.5 Nitrogen Supplements

Four different types of nitrogen sources were used to supplement the fermentation medium which are Yeast Extract (Becton, Dickinson and Company), Peptone (Becton, Dickinson and Company), Ammonium Sulfate (Sigma-Aldrich Company, USA) and Urea (Sigma-Aldrich Company, USA). These nitrogen supplements were acknowledged to enhance the production of bioethanol as reported elsewhere (Sridee *et al.*, 2011; Wang *et al.*, 2012; Protein Sources, 2015).

3.1.5.1 Yeast Extract

Yeast extracts is made up of the concentrates water-soluble portion of *Saccharomyces cerevisiae* cells that have been autolyzed. It is considered as non-animal product and used extensively for many ethanol fermentation purposes (Becton, Dickinson and Company).

3.1.5.2 Peptone

Peptone is natural sources of amino acids and protein which can be obtained from enzymatic digestion or acid hydrolysis of natural products of animal tissue, milks or plants (Protein Sources, 2015).

3.1.5.3 Ammonium Sulfate, $(NH_4)_2SO_4$

Ammonium salts is soluble in water, yielding a clear and colourless solution. *S. cerevisiae* can utilize ammonium ions which can be directly assimilated into amino acids (Sigma-Aldrich Company, USA).

3.1.5.4 Urea

Urea is a principal protein metabolite and major product of removal of free ammonia (NH_4^+) *in vivo*. Urea is soluble in water and other types of solvent; 95% alcohol, absolute alcohol, glycerol and methanol. In ethanol fermentation, urea is the least expensive nitrogen source for yeast growth (Sigma-Aldrich Company, USA).

3.2 Experimental Methods

3.2.1 Preparation of Substrates

The dried sago fibre was ground to pass a 1 mm screen prior to compositional analysis. Figure 15 shows the sago fibre powder (SFP) before it was being used as raw material for glucose production. Dried SFP were then analysed for moisture content prior for enzymatic hydrolysis (AOAC, 1990).



Figure 15: Sago fibre powder (SFP); used for hydrolysis and other analysis

3.2.2 Enzymatic Hydrolysis of Sago Fibre Powder (SFP)

A detail of this enzymatic hydrolysis is shown in Figure 16. Initially, a mixture of sago fibre powder, 7% (w/v) substrate load was prepared in 0.1M KH_2PO_4 (monopotassium phosphate buffer solution) at pH 4 (Kumoro *et al.*, 2008). According to Awg-Adeni *et al.* (2013), the substrate load at 7% (w/v) of SFP was seen to be suitable for hydrolysis process with respect to the gelatinization process as well as saccharification stage as compared to others (5%, 9%, 12% and 15%). The mixture was then gelatinized at 90 – 100 °C for about 30 minutes. After gelatinization process, Liquozyme® SC DS enzyme (Novozymes, Denmark) at 3.4 U/mL

was added into the suspension for another 30 minutes in liquefaction stage. The suspension was stirred constantly to ensure proper mixing of the substrate and enzymes.

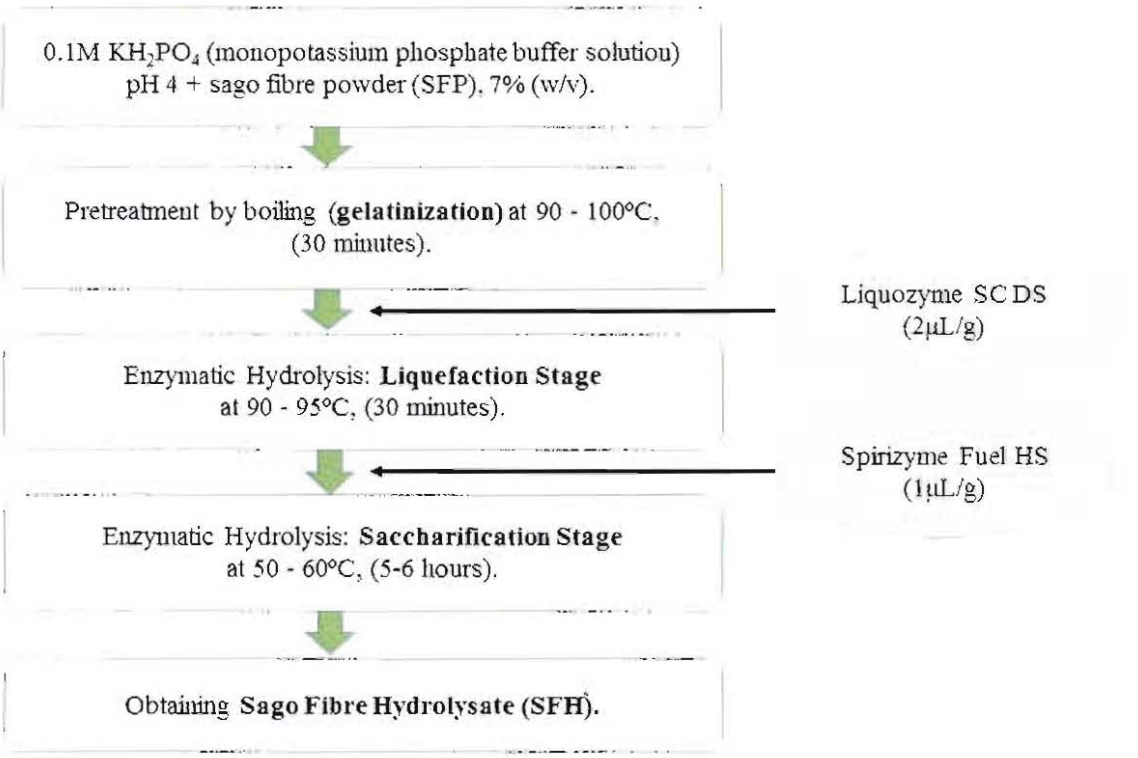


Figure 16: A typical process for the enzymatic hydrolysis of sago fibre powder (SFP) (Adapted from Awg-Adeni *et al.*, 2013)

Then, the suspension was left and subsequently cooled down to 50-60 °C. Spirizyme Fuel HS enzyme (Novozymes, Denmark) was added into the mixture for saccharification stage and was left inside the incubator shaker for another 5-6 hours. Upon completion of the hydrolysis process, the suspension inside the Schott bottle was placed into an ice-water bath to prevent further hydrolysis. The slurry obtained was then filtered from its solid residual fibre by filtration using a 100-mesh sieve filter and being centrifuged at 28, 979 x g for 10 minutes at 4 °C. Figure 17 represents the filtered hydrolysate, named as sago fibre hydrolysate (SFH) which was harvested, stored at 4 °C and further analysed for glucose content using HPLC (Shimadzu, Japan). In order to maximize the glucose concentration as

well as its recovery, recycle method was adopted in this study as reported earlier by Awg-Adeni *et al.* (2013).



Figure 17: Sago Fibre Hydrolysate (SFH) obtained upon centrifugation process

3.2.3 Preparations of Growth Medium

All media were prepared in triplicates and performed aseptically in a laminar air flow cabinet (ERLA Series).

3.2.3.1 Potato Dextrose Agar (PDA)

PDA agar were prepared using 39 g Bacto ™ PDA powder, dissolved in 1000 mL distilled water and sterilized at 121 °C for 15 minutes, left to cooled to about 50-60 °C and then poured into sterile Petri dishes.

3.2.3.2 Yeast Malt Broth (YMB) Medium

YMB medium was prepared by dissolving 1.05 g of Sigma-Aldrich ™ YMB powder in 50 mL shake flask of distilled water. The pH of the inoculum was initially set at 5.5-5.6, before being sterilized at 121 °C for 15 minutes (Vincent *et al.*, 2015).

3.1.3 Commercial Enzymes for Hydrolysis of Sago Fibre

Two types of enzyme (NOVOZYME, Denmark) Liquozyme® SC DS and Spirizyme Fuel HS were used for enzymatic hydrolysis process. Liquozyme SC DS is an α-amylases enzyme, used in our research and reported elsewhere (Zhao *et al.*, 2009; Lareo *et al.*, 2013). Spirizyme Fuel HS is a mixture of glucoamylases which aids in the saccharification stage, hydrolysing 1, 4 and 1, 6 alpha linkages; helps to liberate glucose for subsequent fermentation by yeast (Ramchandran *et al.*, 2015).

3.2.4 Preparation of Microorganism

All media were prepared in triplicates and performed aseptically in a laminar air flow cabinet (ERLA Series).

3.2.4.1 Activation and Culture Preparation of Baker's Yeast

An instant dry baker's yeast (*S. cerevisiae)* labelled as Mauripan was successfully cultured by inoculating 1g of yeast grains in sterile flasks containing 50 mL Yeast Malt Broth (YMB) medium (Figures 18a and 18b). The flasks were left overnight on a rotary shaker (0.76-0.82 x g) at ambient temperature (Vincent *et al.*, 2015). Next, the culture was streaked onto Potato Dextrose Agar (PDA) plates to test for growth and viability (Bellissimi and Ingledew, 2005). These plates were incubated at 37 °C for 24 hours following which single colonies formed from the agar were selected for preparation of inoculum.

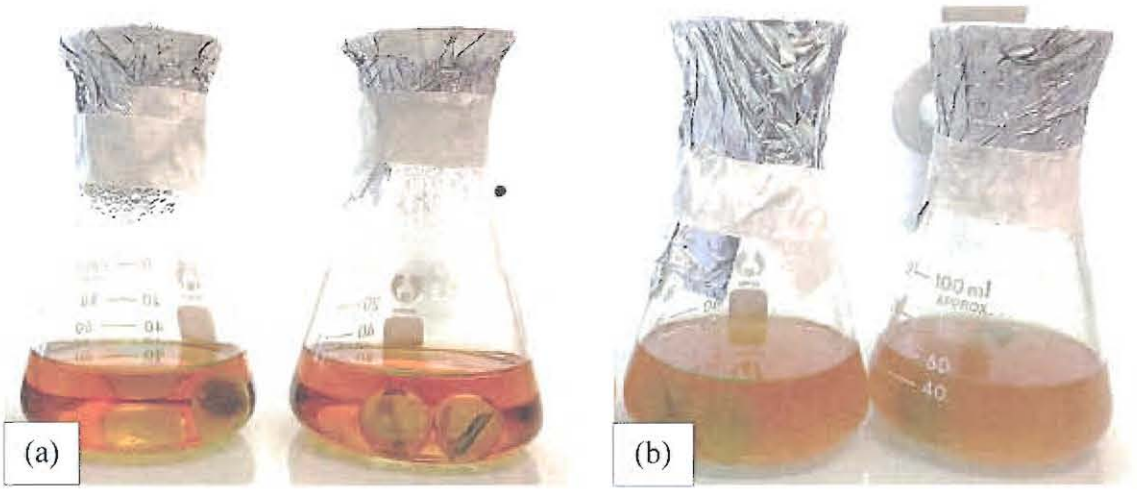


Figure 18: Growth medium for CBY. Sterile YMB medium (a) and YMB medium inoculated with yeasts (b)

3.2.4.2 Preparation of Inoculum Medium & Microorganisms

All inoculum was prepared using one loop of the single yeast colony from the PDA agar and inoculated into 100 mL sterile inoculum media which contains 20 g/L of glucose (commercial glucose) and 5 g/L of yeast extract (as shown in Figure 23). Then, the inoculum flask was incubated in an Orbital Incubator Gyromax$^{TM\ 706}$ Shaker (Hotech Instruments Corp.) at 30 °C with agitation controlled at 0.28 x g for 6-7 hours. Once the OD of yeast cell reached 0.5 to 0.6, which indicates log phase of the yeast growth profiles, the baker's yeast was developed enough and ready to be centrifuged (28, 979 x g for 5 minutes) to obtain the yeast pellet. Lastly, the yeast pellet was ready to be inoculated into sterile fermentation media.

3.2.5 Preparation of Fermentation Media

The study of bioethanol production was based on three criteria; different types of sugars, different concentration of sugars and different types of nitrogen supplement (at a fixed concentration). Sago Fibre Hydrolysate (SFH) was used as alternative and cheaper sugar substrate compared to Commercial Glucose (CG) at the same concentration of glucose. The fermentation medium was supplemented with different types of nitrogen sources in order to observe the fermentation performance and ability of the yeast cells to produce bioethanol from these sugars. SFH and CG media without the addition of any nitrogen sources was used as control in all experiments.

3.2.5.1 Production of Bioethanol by Shake-Flask Fermentation using Sago Fibre Hydrolysate (SFH) and Commercial Glucose (CG) by CBY

All batch fermentation trials (as shown in Figure 19 and Figure 20) were conducted in triplicates using 250 mL shake flasks at 100 mL working volume, initial pH of 5.5-5.6. The temperature was set at 30 °C with agitation controlled at 0.28 x g for all trials.



Figure 19: Shake- flasks fermentation of SFH for bioethanol production

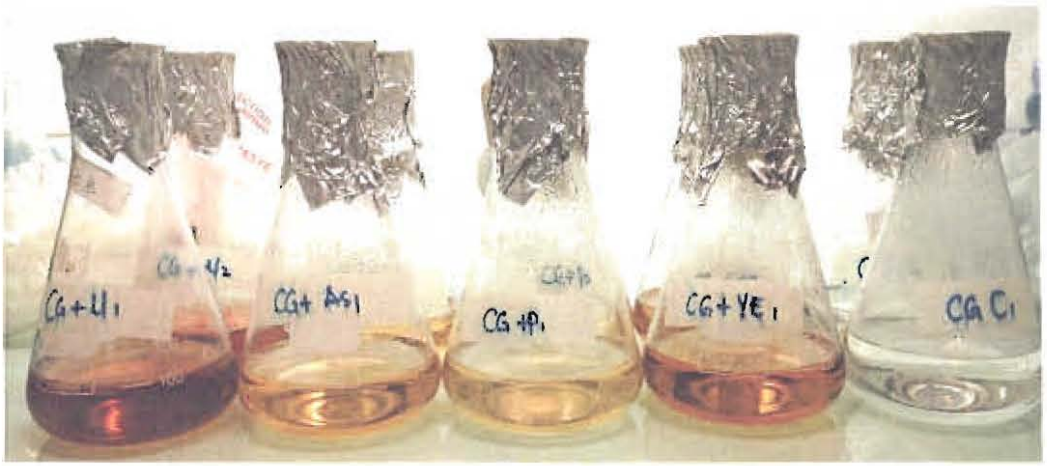


Figure 20: Shake- flasks fermentation of CG media amended with different nitrogen sources for bioethanol production; CG + U: CG amended with Urea; CG + AS: CG amended with Ammonium Sulfate; CG + P: CG amended with Peptone; CG + YE; CG amended with Yeast Extract; CG C: Control media

According to Awg-Adeni (2015), initial glucose of 100 g/L from SFH was the optimum concentration for maximum generation of ethanol compared to 80 g/L, 150 g/L and 270 g/L. However, SFH with initial glucose of 100 g/L cannot be promoted as the sole fermentation media due to several factors. Thus, in this study, lower initial glucose of 50 g/L and 80 g/L (either SFH or CG) were chosen as the substrate concentration; ensuring that maximum ethanol would be generated. The fermentation media were supplemented with different types of nitrogen supplements at fixed concentrations; Yeast Extract, (3 g/L), Peptone, (1 g/L), Ammonium Sulfate, $(NH_4)_2SO_4$, (1.4 g/L) and Urea, (0.8 g/L) as shown in Table 6 with standard fermentation parameters as described before.

Table 6: Media compositions for ethanol fermentation using either glucose from Sago Fibre Hydrolysate (SFH) or Commercial Glucose (CG) as substrate

Trials	Nitrogen Sources	Concentrations of SFH or CG
1.	None (control)	50 and 80 g/L
2.	Yeast Extract (3 g/L)	50 and 80 g/L
3.	Peptone (1 g/L)	50 and 80 g/L
4.	Ammonium Sulfate (1.4 g/L)	50 and 80 g/L
5.	Urea (0.8 g/L)	50 and 80 g/L

Samples were obtained at every 6 hours and then promptly centrifuged at 28, 979 x g for about 10 minutes. The supernatant was then used for the determination of ethanol concentration and glucose consumption by High Performance Liquid Chromatography system (Shimadzu Corporation, Japan). The data obtained from series of fermentation process was compiled and analysed for ethanol yield ($Y_{p/s}$, g/g), ethanol volumetric productivity (Q_p, g/Lh) and ethanol fermentation efficiency (E_f, %). The outcomes were analysed based on these three different criteria to observe which combination would produce the highest bioethanol yield, efficiency and productivity.

3.2.6 Analytical Methods

3.2.6.1 Proximate Analysis of Sago Fibre

Moisture content analysis of sago fibre (SF) was analysed by using standard oven- drying method at 105 °C to constant weight (AOAC, 1990). Analyses of crude fibre, crude fat, total ash and energy were sent to the Veterinary Diagnosis Laboratory, Sarawak.

3.2.6.2 Starch Content Analysis

The starch content of sago fibre (SF) was determined by using iodine starch colorimetric method (Nakamura, 1981). Initially, the gelatinized starch from sago fibre suspension was heated gradually until 80 °C. Then, the gelatinized starch samples were separated from the solid residual fibre by filtration method. The reaction mixture of 1 mL of sample, 0.1 mL Iodine solution (Appendix A) and 8.9 mL distilled water was mixed in a test tube. Then, the absorbance of reaction mixture was analysed by using UV mini-1240v Spectrophotometer (Shimadzu Corporation, Japan) at 590 nm wavelength. The standard curve of starch was first being plotted with various starch concentrations (0.0-1.0 g/L, Appendix B).

3.2.6.3 Total Reducing Sugars Analysis

Reducing sugar analysis obtained from the hydrolysis process was evaluated using the Dinitrosalicyclic acid (DNS) method as described by Miller (1959). The mixture of the 3 mL DNS Reagent (Appendix A) and 3 mL samples were boiled for 15 minutes and cooled before adding with 1 mL of Rochelle salts. The concentration of sugar in the sample is measured by using UV mini-1240v Spectrophotometer (Shimadzu Corporation, Japan) at 575 nm wavelength. From the absorbance reading, sugar content was derived as follows:

$$\text{Sugar (g/L)} = \frac{\text{Absorbance of diluted sample} \times \text{dilution factor (df)}}{\text{M value of standard curve}}$$

The amount of sugar concentration over the amount of sample per litre is calculated as dextrose equivalent (DE) defined as percentage of reducing sugar present on a dry solid basis using the formula:

$$\text{Glucose conversion (\%)} = \frac{A}{B} \times 100\ \%$$

Where, A = concentration of glucose

B = amount of samples per litre

3.2.6.4 Glucose Determination

Glucose content in both sago fibre hydrolysate (SFH) and fermentation broth were analysed via High Performance Liquid Chromatography (HPLC) by using a Shimadzu Chromatographic System (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT and Shimadzu RID-10A. Prior to analysis purpose, the samples was centrifuged at 10, 000 x g for about five minutes, filtered using 0.2 μm Puradisc filter membrane and kept as 4 °C before injected into an Aminex Fermentation Monitoring Column (Aminex HPX-87H

column, 150 x 7.8 mm, BioRad Laboratories, Inc) kept at 60 °C. The mobile phase used was 5 mM H_2S0_4 with a flow rate set at 0.8 mL/min. Standard samples were then prepared using different known concentrations (in % w/v) of all components of interests, such as glucose, xylose, acetic acid, lactic acid and ethanol.

3.2.6.5 Ethanol Determination

Ethanol present in fermentation broth were determined by High Performance Liquid Chromatography (HPLC) using a Shimadzu Chromatographic System (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT and Shimadzu RID-10A. Prior to analysis purpose, the sample was centrifuged at 10, 000 x g for five minutes, filtered using 0.2 μm Puradisc filter membrane and maintained at 4 °C before injected into an Aminex Fermentation Monitoring Column, (Aminex HPX-87H column, 150 x 7.8 mm, BioRad Laboratories, Inc) maintained at 60 °C. The mobile phase used was 5 mM H_2SO_4 with a flow rate set at 0.8 mL/min. Based on the glucose and ethanol profiles, the kinetic parameters for all fermentation profiles were calculated and evaluated based on the following equations (Laopaiboon *et al.*, 2008).

$$Y\ p/s = \frac{p}{s}$$

Y_{ps} = ethanol yield

P = actual ethanol produced (g)

S = glucose utilized (g)

$$Qp = \frac{P}{t}$$

Qp = volumetric ethanol productivity

P = actual ethanol concentration (g/L)

t = fermentation time (h) giving the highest ethanol concentration

$$Ef = \frac{\text{Y ps x 100}}{0.511}$$

Ef = ethanol fermentation efficiency

Y_{ps} = ethanol yield

0.511 = maximum theoretical ethanol yield of glucose consumption

3.2.6.6 Biomass Concentration (Dry Cell Weight)

Ten mL of each sample were centrifuged at 10, 000 x g for 15 minutes and kept at 4 °C, then the clear supernatant was harvested and analysed for glucose and ethanol content. The yeast cells were suspended in sterilized distilled water and centrifuged again at 10, 000 x g for 15 minutes at 4 °C following which the cell free water is discarded, and the pellet were oven-dried at 60 °C overnight until the weight is constant. After drying, the centrifuge tube was reweighed (Lin *et al.*, 2012) and dry cell weight (DCW) was determined as follow:

$$\text{DCW, g/L} = \frac{\text{(wt. of dried centrifuge tube + cells) g - (wt. of centrifuge tube) g x } 10^3}{\text{Sample volume (mL)}}$$

3.2.6.7 Scanning Electron Microscopy (SEM) of CBY

CBY labelled as Mauripan was activated overnight in a sterile Yeast Malt Broth (YMB) medium as described previously in methodology (Section 3.2.4.1). After 24 hours, 500 µL of yeast culture was harvested and centrifuged at 5, 500 x g, at 4 °C for 5 minutes. The supernatant was then removed in order to get the yeast pellet. By using the yeast sample prepared earlier, the cell pellet was mixed with 2.5% glutaraldehyde in distilled water at room temperature for 30 minutes. Next, the tube was centrifuged again at 10, 000 x g for

about 3 minutes. The clear supernatant was removed again, and the sample is washed with distilled water at room temperature, vortexed and left for about 5 minutes. This step was repeated until third times. Then, the yeast pellet was dehydrated with 25%, 50%, 75%, 95% and 100% of ethanol, vortexed and centrifuged at 8, 000 x g for about 2 minutes. The sample was then placed back into a new Eppendorf tube and left to dry overnight at room temperature, with opened cap and covered with aluminium foils to ensure the yeast pellet cells is completely dried. Then, the yeast sample was attached to a circular specimen stub coated with platinum for SEM observation. The physical structure of commercial baker's yeast (Mauripan) was then viewed via JEOL, JSM-6390LA Scanning Electron Microscopy (SEM), Faculty of Resource Science and Technology, Universiti Malaysia Sarawak.

3.2.7 Statistical Analysis

All the data obtained were calculated for the mean and standard deviation. One-way ANOVA ($\alpha = 0.05$) was used to analyse the data and value less than 0.05 was considered as significant. Significant data was analysed with F-distribution to compare means between treatments. Significant difference between treatments was obtained if p value is less than 0.05. LEAD Technologies, MINITAB Version 17.1.0 was used to perform the statistical analyses.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preparation of Sago Fibre (SF) for Fermentable Sugars Production

4.1.1 Characterization of the Sago Fibre (SF)

The characteristic of sago fibre powder (SFP) was presented in Table 7. The amount of residual starch in sago fibre (SF) was highest from CL Nee (Sibu) at 70%, followed by Hup Guan (Johor, 65%) and Ubom (Mukah, 60%). In this study, the residual starch content in dried sago fibre (SF) was 59-60% (on a dry weight basis), which was almost comparable to the starch contents reported by previous studies, in the range of 30 to 70% (Abd-Aziz, 2002; Kumoro *et al.*, 2008; Linggang *et al.*, 2012; Awg-Adeni *et al.*, 2013).

Table 7: Compositional analyses of the sago fibre (as percentage dry weight)

Researchers	**This study (2017)**	Abd-Aziz (2002)	Kumoro *et al.* (2008)	Linggang *et al.* (2012)	Awg-Adeni *et al.* (2013)
Sources of sago fibre	Ubom Mukah	Hup Guan Johor	CL Nee Sibu	Herdsen Pusa	Herdsen Pusa
Residual Starch	59-60	65.70	70.00	58.00	30-45
Moisture	10.43	5.91	6.10	4.90	5-7
Total Ash	3.35	4.10	4.16	3.90	3-4
Total Fibre	22.94	14.80	18.76	32.2	30-35
Protein	3.56	1.00	0.98	1.00	1.00

Note: Apart from Hup Guan in Johor, all other factories are in Sarawak.

The amount of residual starch observed (59- 60%) in sago fibre was quite high as compared to other studies due to several factors. The sago fibre is sourced from different sago mills,

thus different methods or degree of starch extraction together with different facilities contribute to the different quality of the starch extraction process. Most large sago mills have between 10 to 12 extractors which efficiently separate dissolved starch from the fibre. Smaller mills have lesser number of extractors, hence higher percentage of starch in the effluent and attached to the fibres. From the results, residual starch fibres were much reduced at Herdsen (Pusa) from 2012 at 58% to 30- 45% in 2013 due to recycling of sago effluent to remove excess starch prior to disposal into the river (Figure 21). Obviously, in modern factories with efficient extraction facilities, sago fibre contains less starch at 30-45%.



Figure 21: Recycling of sago effluent in a trommel screen at Herdsen Sago Mill (Pusa, Sarawak) helps to reduce residual starch from sago fibre prior to disposal into the river

Higher percentages of residual starch in sago fibre (SF) exhibited a less efficient starch extraction procedure in these factories, which leads to profit loss, disposal of large quantities of starch into the river and eventually leads to environmental pollution. This demands prompt and effective utilisation of the sago fibre to minimise pollution and processing it into

beneficial products to generate side-incomes for these sago mills. Moreover, the amount of residual starch and other composition including free sugars differ from every sago processing mill, depends on the effectiveness of the starch harvesting, extraction, filtration and sedimentation system used (Janggu and Bujang, 2009). Another study pointed out that some of the sago mills applied an advanced and rapid filtration process with greater improvements in starch recovery which then subsequently reduced the amount of raw starch released into the nearby rivers. According to Wischmann *et al.* (2007), normally in laboratory scale or industrial scale, the process of starch granules isolation was also affected by the morphology and molecular composition which leads into different starch physical properties. Hence, the amount of residual starch that still embedded within the sago fibre, would also being influenced by all the differences practices or techniques in sago factories.

4.1.2 Enzymatic Hydrolysis of Residual Starch in Sago Fibre (SF) for Production of Glucose

Enzymatic hydrolysis of sago fibre powder (SFP) into sago fibre hydrolysate (SFH) was done at 2 L scale. The substrate load of SFP at 7% (w/v) was chosen as the optimum substrate loading, resulting in higher concentrations of glucose obtained from residual starch of the sago fibre. In this study, residual starch which embedded within the fibrous matrix component of sago fibre was extracted by boiling method. Boiling at 30 minutes has showed equivalent performance as steaming, which ultimately provide more economical approach for the treatment of starchy-lignocellulosic waste of sago fibre (Janggu and Bujang, 2009). Initially, the suspension of sago fibre powder (SFP) was boiled for 30 minutes, which then produced a highly-viscous liquid paste or commonly known as gelatinization stage. The

gelatinization stage (Figure 22) allows enzymes to penetrate easily into the complex starch structures (Devereux *et al.*, 2011).



Figure 22: Sago fibre powder (SFP) undergo gelatinization stage for 30 minutes

Apart from that, all the hydrogen bonds were broken, and thus crystalline structures were disrupted during gelatinization stage (Wandee *et al.*, 2011). The addition of Liquozyme SC DS (2 µL/g of SFP) to the gelatinized sago fibre suspension resulted in a runnier solution within 15-30 minutes of liquefaction reaction. Then, Spirizyme Fuel HS (1 µL/g of SFP) was added during saccharification stage to accomplish the glucose conversion process. Referring to Hii *et al.* (2012), partly hydrolysed amylose and amylopectin molecules are depolymerised in saccharification stage by glucoamylase action which eliminates the glucose units from non- reducing chain ends.

According to the profile of residual starch used in this study (59-60 %), it is expected to obtain 59 grams of starch for every 100 gram of Sago Fibre Powder (SFP). Theoretically in starch conversion, 1.0 gram of starch and water would generate about 1.11 g of glucose. Thus, from 70 g of sago fibre powder (SFP), it was expected that (41.3 - 42.0 g) of starch

can be extracted upon completion of hydrolysis process. The conversion rate of SFP into SFH obtained was expressed in terms of glucose concentration (g/L) and percentage of yield (%). The efficiency of enzymes in hydrolysing the remaining starch of sago fibre was determined and validated through HPLC analysis for the presence of the main monomeric end products from starch. Table 8 shows the glucose concentration obtained during first cycle of hydrolysis process.

Table 8: Production of glucose during first cycle of enzymatic hydrolysis of sago fibre (7%)

Sago fibre 7% (w/v)	**Glucose Concentration (g/L)**	**Hydrolysis Yield (%)[a]**
Batch 1	43.81 ± 2.27	53.20 ± 0.01
Batch 2	44.14 ± 2.27	55.18 ± 3.14
Batch 3	50.77 ± 2.27	63.46 ± 0.01

Notes: a= {glucose (g)/sago fibre (g)} x 100%. Data presented are the triplicate experiments with mean ± standard errors (SE).

Based on the results, the production of glucose after first cycle of hydrolysis ranged from 43.81 g/L to 50.77 g/L, with the hydrolysis yield ranged from 53.2% to 63.46%, respectively. However, in this study, the highest concentration of glucose obtained was at 50.77 g/L, which exceeded the theoretical amount of glucose that should be generated (42 g) due to existence of other sugar components such as cellobiose and oligosaccharides. Apart from that, the efficiency of enzymatic hydrolysis process also plays an important role towards the sugar production. Further analysis conducted by using HPLC has revealed that, instead of glucose as the main component, cellobiose and oligosaccharides also presents in SFH upon completion of hydrolysis cycle (Appendix C). Additionally, cellobiose/maltose are the other saccharification products from starchy lignocellulosic materials (Bhaumik and Dhepe, 2015). Study by Apun *et al.* (2000) also reported on the same components of reducing sugars from hydrolysed sago fibre. Figure 23 shows the sugar component detected

in SFH through HPLC analysis, indicated glucose (92%), cellobiose (3%) and other oligosaccharides (5%).

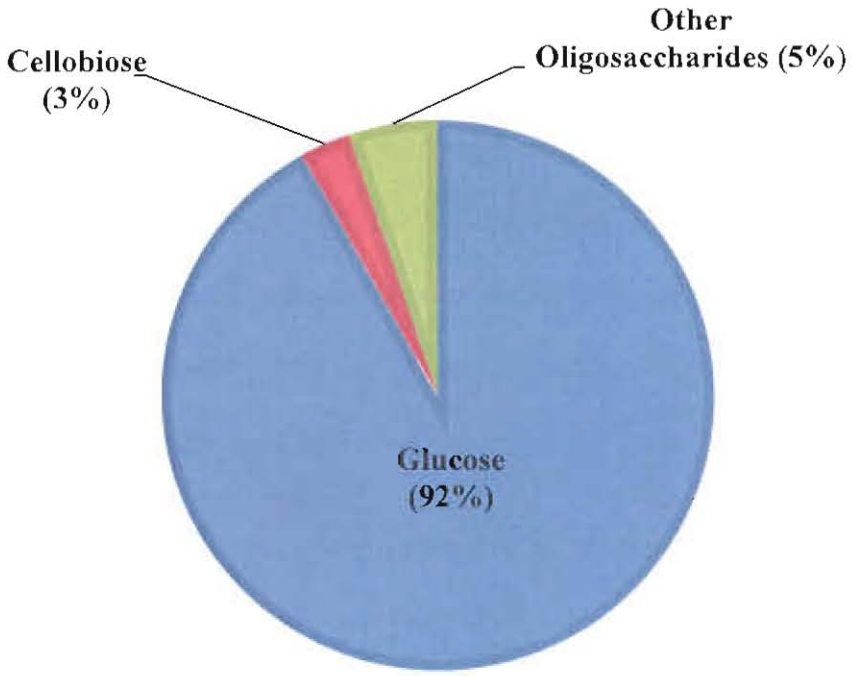


Figure 23: Sugar component in Sago Fibre Hydrolysate (SFH) upon first cycle of hydrolysis process

In this study, Liquozymes SC DS is an endozyme that hydrolyses randomly all the chain of glucose units, thus creating smaller pieces of starch called maltodextrins. Maltodextrin was obtained during liquefaction process contains mainly different oligosaccharides and dextrins (Sunaryanto *et al.*, 2013). The maltodextrin was then converted into glucose units by glucoamylase, which is an exozyme that cleaves off single glucose molecules by hydrolyzing α- (1, 4) linkage from the non-reducing ends of dextrins. During saccharification stage, Spirizyme Fuel HS eventually hydrolyse starch completely to glucose along with, a little maltose and isomaltose e.g maltotriose. Finally, the sugar hydrolysate produced from the hydrolysis process, which mainly contains 92% glucose was used as the sole carbon source for bioethanol production using commercial baker's yeast (CBY), Mauripan. Our findings also demonstrated that, SFP comprised of 22.94% of the total fibre. Lignocellulosic fibre is

mainly composed of cellulose, hemicellulose and lignin. The hydrolysis of sago fibre also indicates other sugars component which derived from other lignocellulosic fibre. Commonly, cellulose is made of glucose monomers, while hemicellulose is made of monomers of pentoses and hexoses (e.g glucose, arabinose, xylose and mannose), (Festucci-Buselli *et al.*, 2007). Hence, sugar derived from the hydrolysis process of sago fibre can be greatly utilized due to its properties as fermentable sugars. Study by Jenol *et al.* (2014) also pointed out the potentials of glucose and other reducing sugars from sago fibre hydrolysate (SFH) and sago pith residue (SPR) hydrolysate as the fermentation medium for production of biohydrogen by *C. butyricum* EB6.

Table 9 represents the glucose concentrations (g/L) of sago fibre hydrolysate (SFH) for three-cycle enzymatic hydrolysis process. The glucose concentration after cycle I of enzymatic hydrolysis was 46.24 g/L. When SFH from cycle I was added with new substrate load for cycle II, 100.10 g/L glucose was generated. At the end of cycle III, maximum amount of glucose obtained was as high as 149.36 g/L. The conversion of glucose was expressed in terms of glucose concentration (g/L) by using HPLC analysis.

Table 9: Glucose production of three- cycle enzymatic hydrolysis of sago fibre

Cycle	Glucose Concentration, (g/L)
I	$46.24^{c} \pm 2.27$
II	$100.10^{b} \pm 2.96$
III	$149.36^{a} \pm 1.53$

*Means with the same letter are not significant at $p<0.05$.

Theoretically, in any chemical reaction, higher substrate concentration would have resulted in higher concentration of end- product. Hence, to maximize the glucose concentration, a three-cycle enzymatic hydrolysis process was adopted on the sago fibre utilizing both

enzymes to obtain high glucose concentration. In every cycle, 7% (w/v) suspension was prepared, thus total substrate load was accounted to be 21% (w/v). Based on this study, it was revealed that conducting hydrolysis process was much easier for cycle II and cycle III because of the solubilisation properties during enzymatic hydrolysis was much better. This is due to the excess enzyme in previous hydrolysis, enabling agitation to be conducted, hence leading to proper distribution of the mass and heat transfer (Awg- Adeni *et al.*, 2013). In this project, it should be noted that all production of bioethanol from sago fibre hydrolysate (SFH) meant that the source of sugar was from hydrolysis of the residual starch that attached to the fibre, and not from cellulosic hydrolysis of the fibre itself.

4.2 Commercial Baker's Yeast (CBY) During Inoculum Stage

4.2.1 General Physical Characteristic of CBY and Its Viability

In order to use CBY as the inoculum, it is imperative to test and observe viability of the cells. A single colony of CBY formed on PDA plates was stained using methylene-blue and examined under compound microscope (at 1000x magnification). Figure 24 showed the compound microscope view of CBY after an incubation period of 24 hours, exhibiting healthy growth of cells. Most of *S. cerevisiae* and baker's yeast cells experienced budding process. Because of its small size, it takes about 30 billion yeast cells to make up to one gram of a compressed baker's yeast inoculum (Abulhassan, 2005).

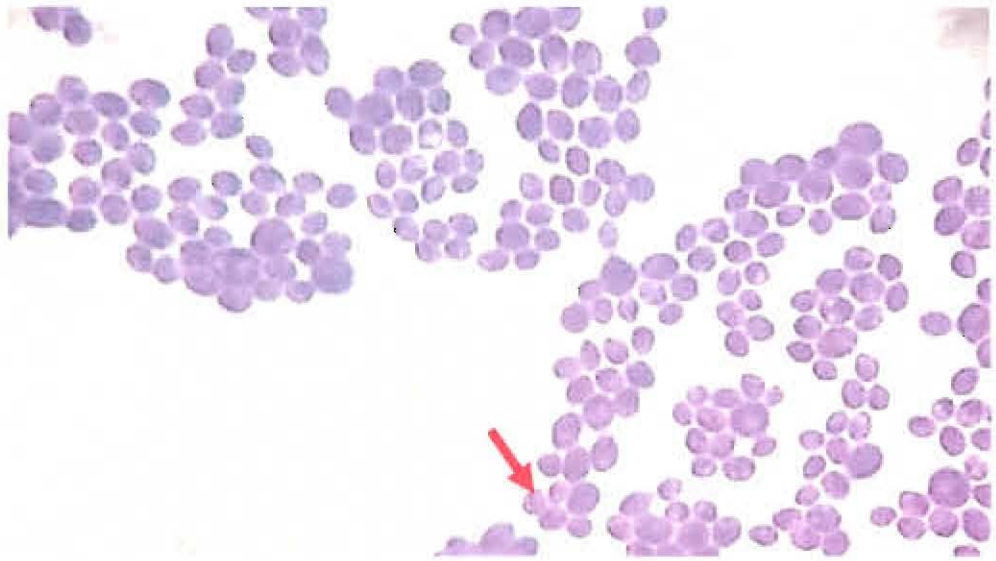


Figure 24: Growth of baker's yeast cells after 24 hours, stained by methylene- blue at 1000x magnification (Arrow shows budding of a yeast cell)

Budding process refer to a process during which a new bud grows from the side of the existing cell wall. This bud eventually breaks away from the mother cell to form a separate daughter cell. Each yeast cell, on average, undergoes this budding process 12 to 15 times before it is no longer capable of reproducing. Under ideal growth environment, a yeast cell reproduces every two to three hours. According to Jayus *et al.* (2016), commercial baker's yeast (CBY) possess several better advantages due to its longer shelf life (over one year), high cells viability (up to 4.6 x 10^{10} yeast cells/g), high efficiency (reduce time and trouble of culture work-up), lower or no microbial contamination, inexpensive price, and superior characteristics in one package (high thermal, sugar, alcohol and acid tolerant) as compared to natural yeast strains. Milkesa (2009) also reported similar justifications on the yeast shelf life, yeast management, population growth, and ethanol productivity produced by natural yeast strains were tended to be lesser than commercial yeasts, which suggested CBY as the superior fermentative microorganism to be used for production of bioethanol via fermentation.

Apart from that, the physical structure of CBY was also observed using Scanning Electron Microscope (SEM) as shown in Figures 25 (a) and 25 (b). The yeast cell (marked with white arrow) has an ellipsoidal shape with a range of diameter between 2.76 - 2.86 μm and length between 3.86 - 4.16 μm. According to Abulhassan and Sayied (2013), the yeast shapes vary from round, oval, elliptical and elongated to sausage-shaped. The cell size also varies according to the species, age of cells and the growth conditions. Commonly the length and diameter of yeast cells lies within 1-10 μm and 1-8 μm, respectively. Others reported that the length vary from 2-10 μm (Mc-Graw Hill, 1997) and the diameter range from 5-10 μm (Boundy-Mills, 2012). Apparently, the cell increases in size as the cell gets older.

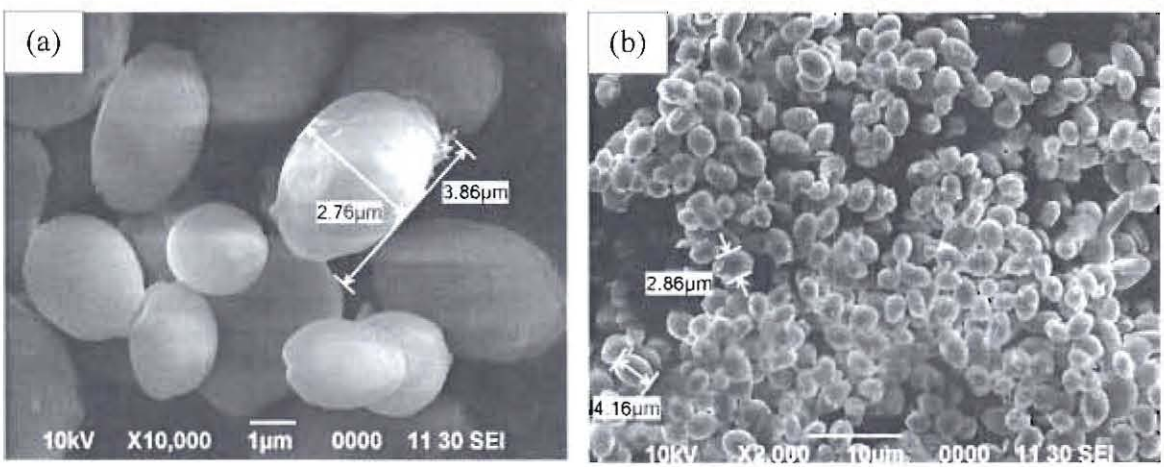


Figure 25: SEM photographs of CBY after 9 hours of incubation, showing close- up view (a) and a colony of cells with various diameter and length (b)

4.2.2 Growth Profile of CBY in Inoculum Media

In Figure 26, the CBY growth profile in inoculum media was analysed before conducting ethanol fermentation. Yeast, like all microorganisms, go through different stages of growth (lag phase, logarithmic phase, stationary phase and death phase (Bellisimi and Ingledew, 2005). When a culture of yeast cells is inoculated into a fresh growth medium, they enter a brief lag phase whereby they are biochemically active but not dividing (Asaduzzaman,

2007). A lag phase occurred over the first 4 hours, which also known as the latency phase (Zamora, 2009). The lag phase refers to the initial growth phase, which number of cells remains relatively constant prior to rapid growth, also known as adaptation time. During this phase, the yeasts are metabolically active, synthesizing ribosomes and enzymes, as well as adapting to their new physical and chemical environment before the cell starts to divide (Bellisimi and Ingledew, 2005). The length of an organism's lag phase is dependent on its ability to adjust to new population size and environmental conditions such as temperature, pH, alcohol, oxygen, salt concentrations, nutrients and others.

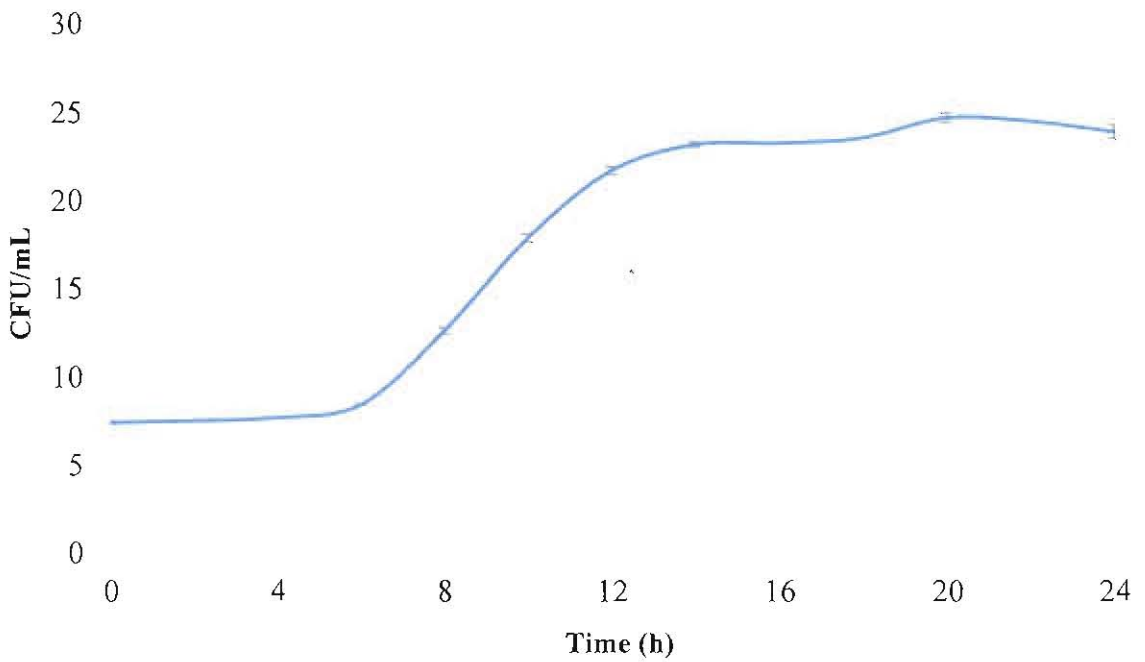


Figure 26: Growth profile of commercial baker's yeast (CFU/mL) in inoculum media. Error bars indicate the mean ± standard deviation of duplicate experiments

After 6 hours, log phase was then started and remain until 12 hours where growth occurred exponentially. In log phase, the yeast begins to grow most rapidly, as they have been introduced with the new environmental conditions. At this stage, the population of yeast cells has increased up to 10^7 to 10^8 cells/mL (Zamora, 2009). After 12 hours of incubation period,

the growth rate began to decline and slightly no change or static conditions was observed. Thus, the baker's yeast was at the stationary phase, or exponential phase. During this stage, cell metabolism become slow and cells stop rapid cell division. According to Russell (2003), high cell density was achieved by allowing cells to grow for longer time to maximize the ethanol production.

Previous studies done by Awg-Adeni *et al.* (2013) with three different incubation periods (6h, 9h and 12h) of inoculums were selected to study their effects on ethanol production performance in hydrolysate of sago fibre (SFH) as its fermentation medium. Based on the results, CBY which had been acclimatized for 9 to 12 hours was discovered to have the shortest lag phase time as compared to pre- incubation times for 6 to 9 hours. In this study, the CBY was chosen to acclimatize for 9 hours which indicates the logarithmic phase of CBY growth profile. During this stage, the OD_{600} of yeast cells reached 0.4 to 0.6, which means the yeast cell was mature enough, ready to be inoculated and transferred into a fresh and new fermentation medium.

4.3 Potential of Commercial Baker's Yeast (CBY) for Ethanol Fermentation

In order to prove the CBY cells was able to metabolize carbon source for generating ethanol, a basic experiment was carried out utilizing 50 g/L of glucose from commercial glucose (CG) without addition of any nitrogen source. The fermentation was conducted in standard parameters; 100 mL working volume, agitated at 0.28 x g, with initial pH of 5.5 and maintain at 30 °C. Figure 27 shows the fermentation profiles, obtained from the experiment.

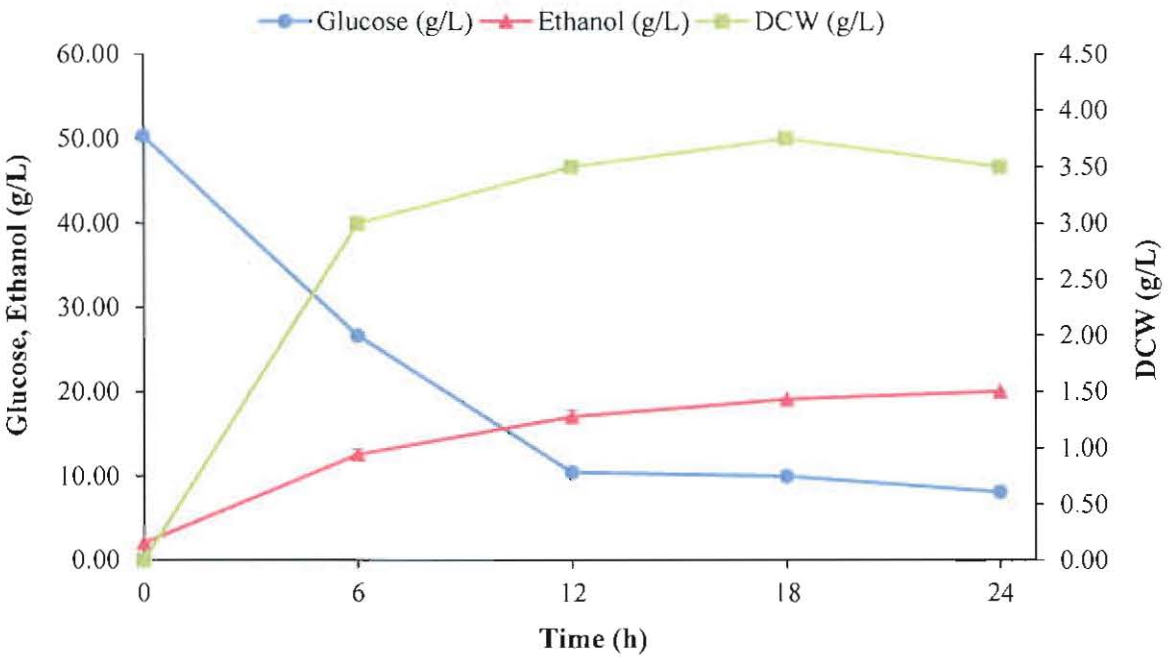


Figure 27: Fermentation profile on bioethanol production by CBY under Commercial Glucose (CG) media (without any addition of nitrogen source)

The graph represents the correlation between CBY growth (DCW), residual glucose and ethanol concentration utilizing 50 g/L glucose in CG media solely. The initial glucose concentration of 50.28 ± 0.14 g/L was reduced to 9.99 ± 0.01 g/L after 18 hours of the fermentation with total glucose consumption of 80.13%. Glucose was then further consumed until it remains to 8.14 ± 0.62 g/L with the total consumption of 83.81% at 24 hours of fermentation and maximum ethanol concentration was 20.04 ± 0.06 g/L. The biomass concentration increased starting from 6 hours to 18 hours, and yet decreased to the end of fermentation period with 3.50 ± 0.00 g/L yields. The fermentation efficiency based on the glucose conversion to ethanol for this fermentation was 93.07 ± 1.41%. Details on glucose consumption, ethanol production and CBY growth profile (DCW) were presented in Table 10.

Table 10: Glucose consumption, ethanol production and dry cell weight (DCW) of CBY in CG media (without addition of any nitrogen sources)

Time (h)	Glucose (g/L)	Ethanol (g/L)	DCW (g/L)
0	50.28 ± 0.14	2.07 ± 0.04	0.00 ± 0.00
6	26.62 ± 0.40	12.58 ± 0.67	3.00 ± 0.00
12	10.47 ± 0.27	17.02 ± 0.79	3.50 ± 0.00
18	9.99 ± 0.01	19.10 ± 0.14	3.75 ± 0.35
24	8.14 ± 0.62	20.04 ± 0.06	3.50 ± 0.00

In overall, CG media without addition of any nitrogen source shows good performance for CBY to grow and capable of generating ethanol. According to this study, maximum amount of ethanol was achieved (20.04 ± 0.06 g/L) during 24 hr of fermentation, thus contribute to high ethanol yield which was 0.48 ± 0.005 g/g. This results already implies the effectiveness of the CBY in metabolizing the glucose, in the fermentation process itself. However, glucose was not completely consumed during 24 hr of fermentation, implying that longer incubation time is required for CBY cell to consume enough glucose and yet, producing more ethanol. Apart from that, the existence of essential nutrients in the fermentation medium might be insufficient enough for the CBY to generate more ethanol. Hence, it is crucial to supplement an adequate amount of nitrogen source in both CG and SFH media, thereby enhanced glucose consumption as well as ethanol production. As mentioned by Chang *et al.* (2011), the free amino nitrogen (FAN) assimilation by baker's yeast cell also can alters its intracellular functionality, resulting in glucose utilization, however without enhancement on the fermentation rate.

4.4 Sago Fibre Hydrolysate (SFH) as a Sole Fermentation Media for Production of Bioethanol using CBY at Initial Glucose of 50 g/L and 80 g/L

In this study, SFH (without addition of any nitrogen source) was used as a sole fermentation media for generating ethanol by using two different initial glucose concentration which were 50 g/L and 80 g/L. This section focused on evaluating the performance of CBY towards glucose consumption and ethanol production profile in SFH, as a sole fermentation media.

4.4.1 Glucose Consumption Profile

Figure 28 represent the fermentation profiles, indicating glucose consumption in SFH fermentation by CBY during 24 until 48 hours of fermentation.

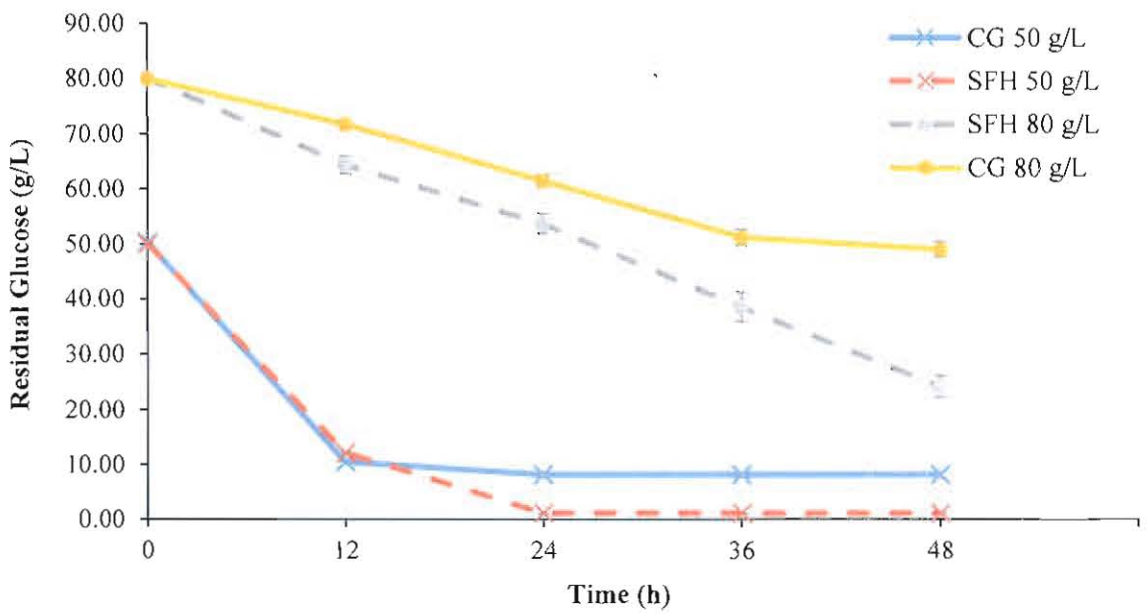


Figure 28: The profile of glucose consumption in SFH fermentation by Commercial Baker's Yeast (CBY) under 50 g/L and 80 g/L initial glucose concentration. Error bars indicate the standard deviations of duplicate experiments

Based on the fermentation profiles, it was observed that, 50 g/L of glucose in SFH media was almost consumed by the CBY during 24 hours of fermentation, with remaining glucose at 1.11 ± 0.01 g/L. Thus, the total glucose consumption in 50 g/L SFH media was 97.78%, which indicate the fastest glucose consumption, compared to 50 g/L glucose in CG media, with total glucose consumption of 83.81%, at 24 hours of fermentation. In contrast to 80 g/L of initial glucose concentration, it was observed that, 80 g/L of glucose in SFH media was not completely consumed by the yeast during 48 hours of fermentation, with remaining glucose of 24.155 ± 1.40 g/L, thus indicates 69.83% of glucose consumption. Similar trend was also observed under 80 g/L of glucose in CG media. The remaining glucose was at (48.99 ± 1.40 g/L), with lower total glucose consumption of 38.75% only. In this condition, 80 g/L glucose in SFH media demonstrated slightly better performance on glucose consumption rather than in 80 g/L glucose of CG media.

According to this study, longer incubation time would be required for the CBY cells to metabolize the glucose, as the initial glucose concentration was increased. Both SFH and CG fermentation under 50 g/L of glucose only requires 24 hours of incubation time prior to complete the fermentation process, whereas under 80 g/L of glucose in both media requires longer than 48 hours of incubation time for the CBY to metabolize the carbon source and yet, generating more ethanol. In this study, same inoculum concentration was introduced for both 50 g/L and 80 g/L media during fermentation process. Awg- Adeni *et al.* (2013) has pointed out that, increasing the yeast concentration did have a significant effect on the fermentation time. However, the maximum ethanol concentration was not influenced by the yeast concentration. In this study, it was obviously shown that similar yeast concentration was used, thus producing maximum ethanol in 50 g/L glucose media only, not in 80 g/L glucose media.

4.4.2 Ethanol Production Profile

Figure 29 represent the fermentation profiles, indicating ethanol production in SFH fermentation by CBY during 24 until 48 hours of fermentation. Based on the results obtained, it was observed that, 50 g/L glucose from SFH media can generate maximum ethanol during 24 hours of fermentation, which was at (20.33 ± 0.15 g/L). Similar ethanol concentration was obtained in 50 g/L glucose of CG media, which was at (20.04 ± 0.06 g/L). However, in 80 g/L glucose of SFH, much lower ethanol was produced (13.32 ± 0.12 g/L, 7% lower) than in 50 g/L SFH media. Similar ethanol profile was obtained under 80 g/L glucose in CG fermentation, which was about 12.98 ± 0. 04 g/L.

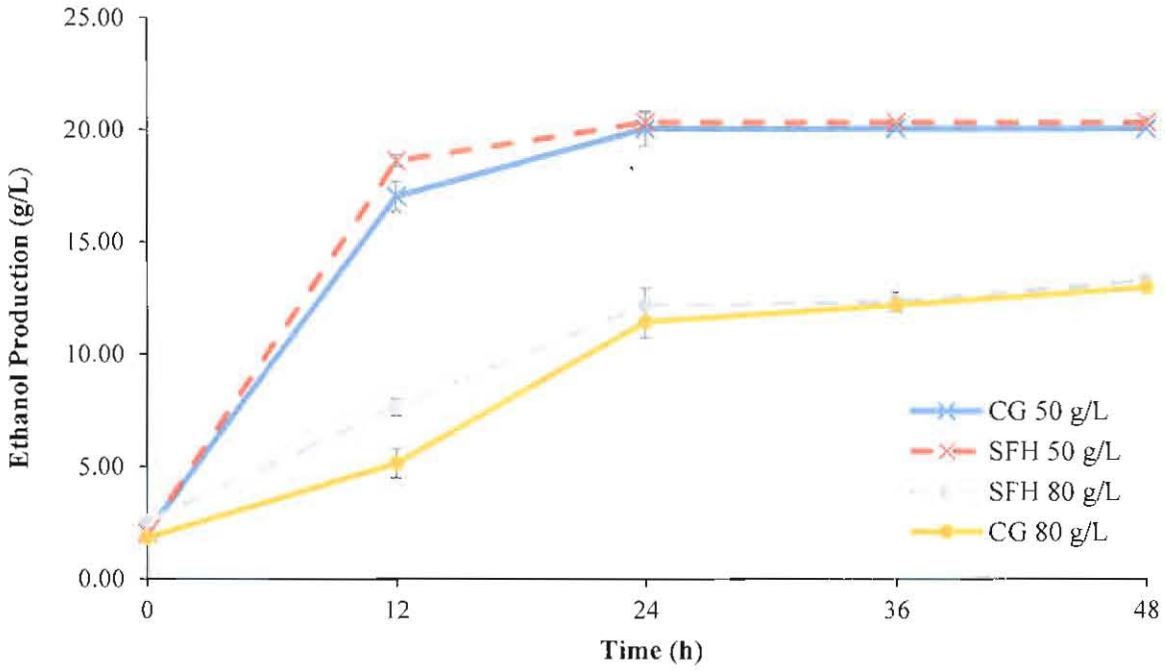


Figure 29: The profile of ethanol production in SFH fermentation by CBY under 50 g/L and 80 g/L initial glucose concentration. Error bars indicate the standard deviations of duplicate experiments

According to the ethanol production profile, higher and maximum concentration of ethanol was achieved when lower initial glucose concentration (50 g/L) was introduced, whereas lower concentration of ethanol was generated when higher initial glucose concentration was

introduced (80 g/L). In this condition, the yeast might be unprotected to severe conditions such as high osmotic pressure, which increased the by- product inhibition and nutritional limitations (Chunkeng *et al.,* 2011). Normally, inadequate of nitrogen source can also cause inhibition towards the yeast growth, thus reduce ethanol conversion, as well as ethanol yield (Shen *et al.,* 2012; Phukoetphim *et al.,* 2017). Table 11 summarizes the important kinetic parameters of the ethanol fermentation utilizing SFH as a sole fermentation media at initial glucose concentrations of 50 g/L and 80 g/L. By comparing both initial glucose concentration, clearly 50 g/L of initial glucose from SFH media shows better performance compared with 80 g/L of initial glucose concentration. In term of glucose consumption profile, SFH media with initial glucose of 50 g/L indicates perfect fermentation, with 97.78% of its total glucose consumption. This condition demonstrated that the CBY completely metabolized the carbon source to generate alcohol within 24 hours of incubation time, even without any supplementation of nitrogen source.

Table 11: Kinetic parameters of ethanol fermentation utilizing SFH as a sole fermentation media at initial glucose of 50 g/L and 80 g/L

Kinetic Parameters	**50 g/L**		**80 g/L**	
	SFH	**CG**	**SFH**	**CG**
Initial Glucose (g/L)	50.00 ± 0.00	50.28 ± 0.14	80.06 ± 0.04	79.98 ± 0.08
Glucose Consumption Efficiency (%)	97.78	83.81	69.83	38.75
Ethanol (g/L)	20.33 ± 0.15	20.04 ± 0.06	13.32 ± 0.09	12.98 ± 0.05
$Y_{p/s}$ (g/g)	0.42 ± 0.003	0.48 ± 0.005	0.24 ± 0.008	0.42 ± 0.011
Q_p (g/Lh)	0.85 ± 0.006	0.84 ± 0.002	0.28 ± 0.002	0.27 ± 0.001
E_f (%)	81.35 ± 0.572	93.07± 0.996	46.65 ± 1.50	82.00 ± 2.250

Notes: Parameters studied: $Y_{p/s}$ ethanol yield (g/g), Q_P ethanol volumetric productivity (g/Lhr), E_f fermentation efficiency (%). All data presented are the average of duplicate experiments with standard errors. Theoretical ethanol yield is 0.511 g/g glucose.

Based on the kinetic parameters, 50 g/L of initial glucose concentration from SFH media shows the highest glucose consumption efficiency (97.78%), higher fermentation efficiency (81.35 ± 0.572%), with respective ethanol yield of (0.42 ± 0.003 g/g), indicating the CBY was well tolerates with SFH media as a sole fermentation media without any addition of nitrogen source. However, once higher initial glucose (>50 g/L) was introduced into the same process, both ethanol yield and fermentation efficiency show some decline on their value, although the fermentation time was prolonged to achieve maximum ethanol concentration. The nitrogen source was also needed if higher initial glucose was introduced (>50g/L), thus the fermentation rate become faster and demonstrated better ethanol fermentability. Usually, the extension of fermentation time is a common practice in high gravity (HG) or very high gravity (VHG) fermentation, which also could decrease the ethanol productivity correspondingly (Li *et al.,* 2009).

Interestingly, in this study, SFH produced from Sago Fibre Powder (SFP) contains numerous nutrients, as well as trace elements, shown in Table 12. The highest is Phosphorous (3663.8 mg/L), followed by Potassium (2840.9 mg/L) and Calcium (101.17 mg/L). Traces of other elements such as Magnesium, Iron, Manganese, Copper, Zinc and Boron were detected as well. This is a great and huge advantages since the fibre is a by-product disposed from sago mills into the river. As mentioned by Walker and Stewart (2016), minerals, especially key metal ions, are often seemed as essential and crucial determinants of yeast fermentation performance, inspite of the fact that the nature and concentration of potassium, sulphur and magnesium are the key examples of 'bulk' minerals needed in millimolar concentrations, whilst calcium, sodium, iron, cobalt, zinc, copper, manganese, nickel and selenium are the trace elements needed in micromolar, or less concentrations (Walker, 2004).

Normally, a complex fermentation medium used for fermented beverages production (i.e malt wort, molasses, wine) must contain adequate levels of inorganic ions for yeast growth, however supplementation with additional minerals may be not necessary. SFH itself, consists high amount of phosphorus content (3663.8 mg/L) due to existing phosphate salts in KH_2PO_4 buffer solution, which was mixed together with SFP during enzymatic hydrolysis process. Phosphorus is essential for biosynthesis of nucleic acids, phospholipids and ATP. The phosphate content of yeast cells is approximately 3-5% of dry weight and this is mainly in the form of orthophosphate (H_2PO_4) which acts as a substrate and enzyme effector (Walker and Stewart, 2016). Potassium is also important ion that affects the growth and survival of living cells as been reported by Safaei *et al.* (2016). Moreover, magnesium (Mg^{2+}), calcium (Ca^{2+}), and zinc (Zn^{2+}) were also believed to encourage sugar conversion rate as well as metabolic changes in yeast biomass growth (Aleksander *et al.*, 2009).

Magnesium ion can modulate the electrical potential across cell membranes, which allows nutrients to pass back and forth. Zinc is particularly significant as cofactor of the terminal alcohologenic enzyme, ethanol (alcohol) dehydrogenase. Manganese is essential trace elements that are necessary by yeast cells in fermentation process for optimal yeast growth. Stehlik -Thomas *et al.* (2004) claimed manganese has significant role in *S. cerevisiae* metabolism as a part of some enzymes, e.g pyruvate carboxylase. Thus, SFH also portrays great advantages, serves as a sole complex fermentation medium due to the existing essential nutrients which ultimately important for the yeast growth and metabolism in ethanol fermentation process. Moreover, the CBY also continued their growth steadily and faster when inoculated into SFH media compared to Commercial Glucose (CG) media. This phenomenon might have some justification as claimed before which promotes the significance of inorganic materials as well as trace elements that exists in SFH media could

contribute to better fermentation performance by baker's yeasts compared with CG media. As claimed by Shafaghat *et al.* (2010), medium compositions have very high effects on robustness and growth of microorganism. Studies by Walker *et al.* (2006) and Pereira *et al.* (2010) concluded that the presence of ionic compounds such as manganese, iron, magnesium, zinc and copper in a complex fermentation media can enhance yeast fermentation by increasing sugar conversion to ethanol, improved final ethanol yield, enriched cell viability and stress tolerance. Thus, these nutrients are beneficial enough in fermentation processes, should SFH is to be used as an alternative substrate to produce bioethanol. SFH could serve as complex fermentation media due to the presence of heterogenous compounds in its hydrolysate, which are very significant in conducting ethanol fermentation process.

Table 12: Nutrient/trace elements analyses in Sago Fibre Hydrolysate (SFH) containing 100g/L of glucose

Nutrients	Concentration (mg/L)
1. Phosphorus, P	3663.8
2. Potassium, K	2840.9
3. Calcium, Ca	101.17
4. Magnesium, Mg	46.78
5. Manganese, Mn	3.51
6. Iron, Fe	0.70
7. Boron, B	0.45
8. Zinc, Zn	0.36
9. Copper, Cu	0.03

4.5 Addition of Yeast Extract (YE) as Nitrogen Source in Sago Fibre Hydrolysate (SFH) for Production of Bioethanol using CBY

This section focused on the results obtained, glucose consumption and ethanol production profiles, based on fermentation process utilizing SFH with the addition of YE (3 g/L) as source of nitrogen at initial glucose concentration of 50 g/L and 80 g/L.

4.5.1 Glucose Consumption Profile in SFH with YE

Figure 30 represented the fermentation profiles, indicating the glucose consumption profile in SFH (with addition of YE) fermentation by CBY during 24 until 48 hours of fermentation.

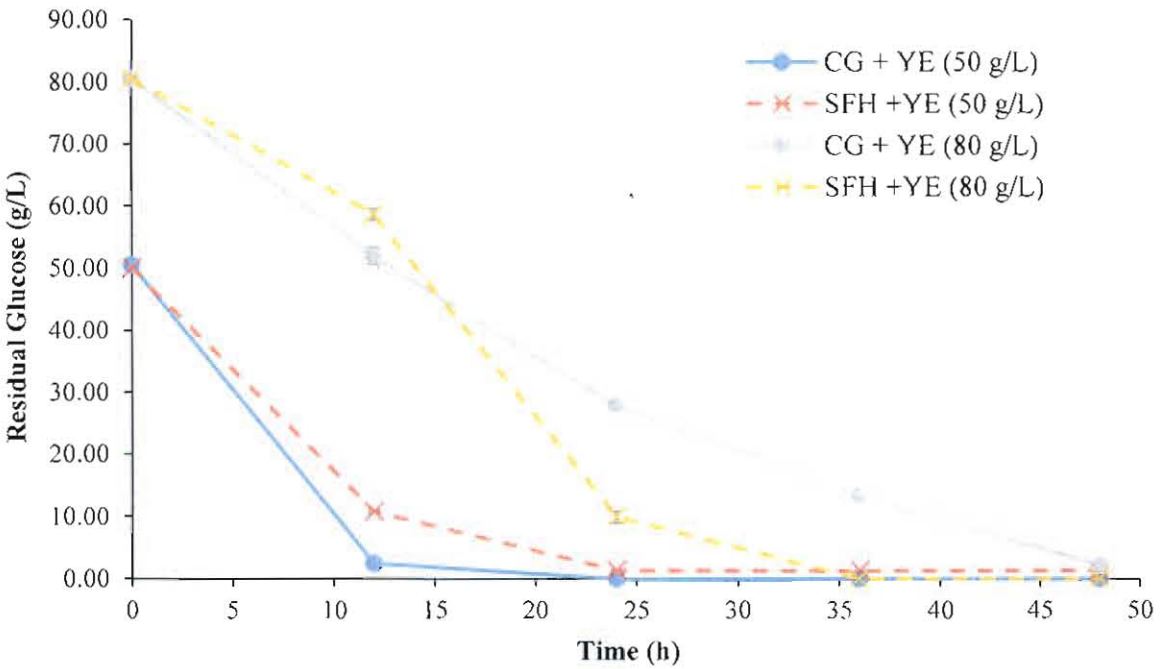


Figure 30: The profile of glucose consumption in SFH (with addition of yeast extract) fermentation by Commercial Baker's Yeast (CBY) under 50 g/L and 80 g/L initial glucose concentration. Error bars indicate the standard deviations of duplicate experiments

Based on the fermentation profiles, it was observed that, 50 g/L of glucose in SFH media was almost consumed by the CBY during 24 hours of fermentation, with remaining glucose at 1.33 ± 0.04 g/L. Thus, indicates 97.34% of glucose consumption, slightly lowest compared to 50 g/L glucose in CG media, with 100% of glucose consumption at 24 hours of

fermentation. In contrast to 80 g/L of initial glucose concentration, glucose in SFH media was fully metabolized by the CBY after 24 hours of fermentation, indicates good performance of CBY when amended with YE and at higher concentration (80 g/L) of glucose. However, in 80 g/L glucose of CG media, the glucose was almost utilized during 48 hours of fermentation, indicates 97.33% of glucose consumption, which is slower compared in 80 g/L glucose in SFH media.

4.5.2 Ethanol Production Profile in SFH with YE

Figure 31 represent the fermentation profiles, indicates ethanol production profile in SFH (with addition of YE) fermentation by CBY during 24 until 48 hours of fermentation.

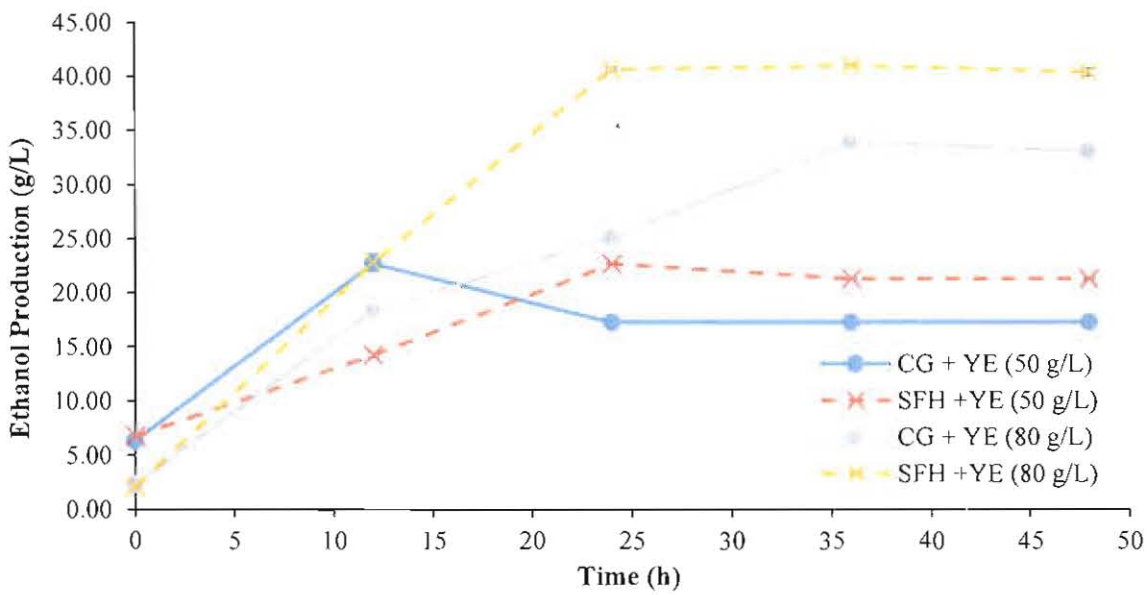


Figure 31: The profile of ethanol production in SFH (with addition of yeast extract) by Commercial Baker's Yeast (CBY) under 50 g/L and 80 g/L initial glucose concentration. Error bars indicate the standard deviations of duplicate experiments

From the results obtained, it was observed that, 80 g/L of glucose in SFH media generate the highest ethanol concentration (41.04 ± 0.04 g/L) and followed by 80 g/L glucose in CG

media (33.96 ± 0.04 g/L), respectively. Both media produced maximum ethanol concentration during 36 hours of fermentation, much earlier than expected time for the fermentation to complete. Similar ethanol performance was obtained in 50 g/L glucose SFH media, highest ethanol achieved was (22.71 ± 0.31 g/L) during 24 hours of fermentation, whereas in 50 g/L CG media, similar amount of ethanol was also generated (22.70 ± 0.30 g/L), during 12 hours of fermentation, which is faster compared to SFH media. Clearly, the addition of YE as nitrogen source, significantly assisting in fermentation process, thus enhanced the ethanol fermentability towards the CBY. Table 13 summarizes the important kinetic parameters of the ethanol fermentation utilizing SFH (with addition of YE as source of nitrogen) at initial glucose concentrations of 50 g/L and 80 g/L.

Table 13: Kinetic parameters of ethanol fermentation utilizing SFH (with addition of YE) at initial glucose concentrations of 50 g/L and 80 g/L

Kinetic Parameters	**50 g/L**		**80 g/L**	
	SFH + YE	**CG + YE**	**SFH + YE**	**CG + YE**
Initial Glucose (g/L)	50.00 ± 0.00	50.53 ± 0.02	80.54 ± 0.44	80.51 ± 0.41
Glucose Consumption Efficiency (%)	95.80	95.03	100	83.24
Ethanol (g/L)	22.71 ± 0.31	22.70 ± 0.30	41.04 ± 0.04	33.96 ± 0.04
$Y_{p/s}$ (g/g)	0.47 ± 0.005	0.47 ± 0.002	0.51 ± 0.002	0.51 ± 0.001
Q_P (g/Lh)	1.26 ± 0.017	1.89 ± 0.025	1.14 ± 0.001	0.94 ± 0.001
E_f (%)	92.77 ± 1.061	92.51 ± 0.336	99.71 ± 0.460	99.15 ± 0.109

Notes: Parameters studied: $Y_{p/s}$ ethanol yield (g/g), Q_P ethanol volumetric productivity (g/Lhr), E_f fermentation efficiency (%). All data presented are the average of duplicate experiments with standard errors. Theoretical ethanol yield is 0.511 g/g glucose.

Referring Table 13, both SFH and CG media (50 g/L and 80 g/L glucose) portrays similar performance in terms of ethanol yield, as well as its fermentation efficiency. By comparing initial glucose concentration of 50 and 80 g/L in SFH media, the $Y_{p/s}$ (g/g) and E_f (%) were improved from 0.47 ± 0.005 g/g to 0.51 ± 0.002 g/g, and 92.77 ± 1.061 % to 99.71 ± 0.460 %. Similar performance was also obtained in CG media, indicates the addition of YE would enhanced the fermentation rate, thus higher ethanol yield was obtained. Interestingly, the glucose consumption rate in 50 g/L shows similar results, except in 80 g/L glucose from SFH with YE, which is 100%. In 80 g/L glucose of SFH and CG media, ethanol was maximum during 36 hours of fermentation.

In overall, based on the findings that has been discussed in Sections 4.4 and 4.5, ethanol fermentation utilizing SFH as the sole fermentation medium was better only at 50 g/L initial glucose concentration. By comparing the glucose consumption in 50 g/L SFH and 50 g/L CG media, both glucose were metabolized during 24 hours of fermentation. Maximum ethanol was achieved, as the glucose were completely utilized by the CBY during the fermentation period. Hence, promoting 50 g/L glucose of SFH media was capable to be used in generating alcohol faster than in 80 g/L SFH media. However, in this study, 80 g/L glucose of SFH and CG media (without addition of nitrogen source) indicates less efficient of ethanol fermentability, although the incubation period was extended for the CBY to completely consume the carbon source.

Due to several limitations, including lack of nitrogen source, glucose was not completely metabolized even after 48 hours of fermentation. According to Lin *et al.* (2012), longer incubation period was required by higher initial glucose concentration, when pH was not controlled during fermentation, which then reduced the ethanol conversion efficiency. In this study, the pH of both media was controlled only during initial stage of fermentation process,

and yet uncontrolled during experiment until it was finished. The pH was decreased from 5.5 to about 3.5- 3.8, after 18 hours of fermentation process and remains constant thereafter in fermentation utilizing yeast extract, peptone and ammonium sulfate as nitrogen sources, whereas in urea supplemented the pH reading was at 4 until the end of fermentation process. As claimed by Wang *et al.* (2012), the differences in pH between all the fermentation media are mainly due to how the nitrogen sources are transported across the cell membrane. The transport of ammonium is directly driven by the electrochemical plasma membrane potential, generated by the plasma membrane ATPase, in which ion is taken up and proton is later excreted to the medium to keep a constant intracellular pH, thus causes pH decline in the extracellular medium (Russell, 2003). Besides, in natural environment, yeasts could use a wide range of nitrogen-containing compound as the sole nitrogen source, but not all are consistently suitable for supporting optimal yeast growth and the fermentative reactions (Barbosa *et al.*, 2012).

Higher initial glucose concentration would lead to longer glucose uptake by the yeast cells, even though the same initial cell concentration was introduced during inoculum preparation. However, with addition of yeast extract (YE) as source of nitrogen, CBY consumed glucose faster rather than in SFH media, solely. Similar pattern was also obtained on the ethanol production, both media were able to generate maximum ethanol concentration earlier at 12 hours, followed by 24 hours and 36 hours of fermentation. Between 50 g/L and 80 g/L glucose from SFH and CG (with addition of YE), 80 g/L SFH media shows efficient fermentation, whereas in 50 g/L of SFH and CG media, also indicates higher ethanol yield, as well as its fermentation efficiency. Very high ethanol concentration was observed in 80 g/L SFH media, thus contribute to high ethanol yield which was at 0.51 ± 0.002 g/g.

Ethanol fermentation utilizing 50 g/L glucose of SFH (with addition of YE) shows enhancement in terms of ethanol yield (0.47 ± 0.005 g/g), as well as its fermentation efficiency (92.77 ± 1.061 %), compared to SFH (without addition of nitrogen source) which is slightly lower in ethanol yield (0.42 ± 0.003 g/g) as well as E_f, (81.35 ± 0.572 %). Hence, from this study, clearly the supplementation of YE enhanced the ethanol fermentability towards the CBY. This study also confirmed that sago fibre hydrolysate (SFH) should be amended with sufficient and correct amount of nitrogen source for the CBY growth and increase the ethanol yield, as well as to enhance the fermentation efficiency and ethanol volumetric productivity of the fermentation process.

4.6 Addition of Selected Nitrogen Sources utilizing Sago Fibre Hydrolysate (SFH) on Ethanol Fermentability using CBY

In this study, three selected low-cost nitrogen sources; Peptone (P), Ammonium Sulfate (AS) and Urea (U) were supplemented with SFH. The profile of glucose consumption, ethanol production as well as growth profile (DCW) of CBY were analysed. The initial glucose of 50 g/L was used throughout the study. All fermentation parameters were similar as reported in Subsection 3.2.5.1.

4.6.1 Effects of Various Nitrogen Sources on Glucose Consumption Profile

Figure 32 shows the results obtained, based on glucose consumption profile of 50 g/L glucose in SFH and CG fermentation. It was observed that, in SFH media, the glucose consumption in all fermentation supplemented by four different nitrogen sources were reduced significantly, and yet during 24 hours of fermentation, the glucose remained between 1.11 ± 0.01 g/L to 4.18 ± 0.09 g/L. This indicated good performance towards CBY in metabolizing the glucose in SFH media, even without and with addition of nitrogen

sources. In this study, 50 g/L of glucose in SFH media shows better performance as raw substrate in generating high amount of alcohols by CBY. Additionally, SFH was proven to have beneficial nutrients which is enough in assisting fermentation processes, should SFH is to be used as an alternative substrate to produce bioethanol.

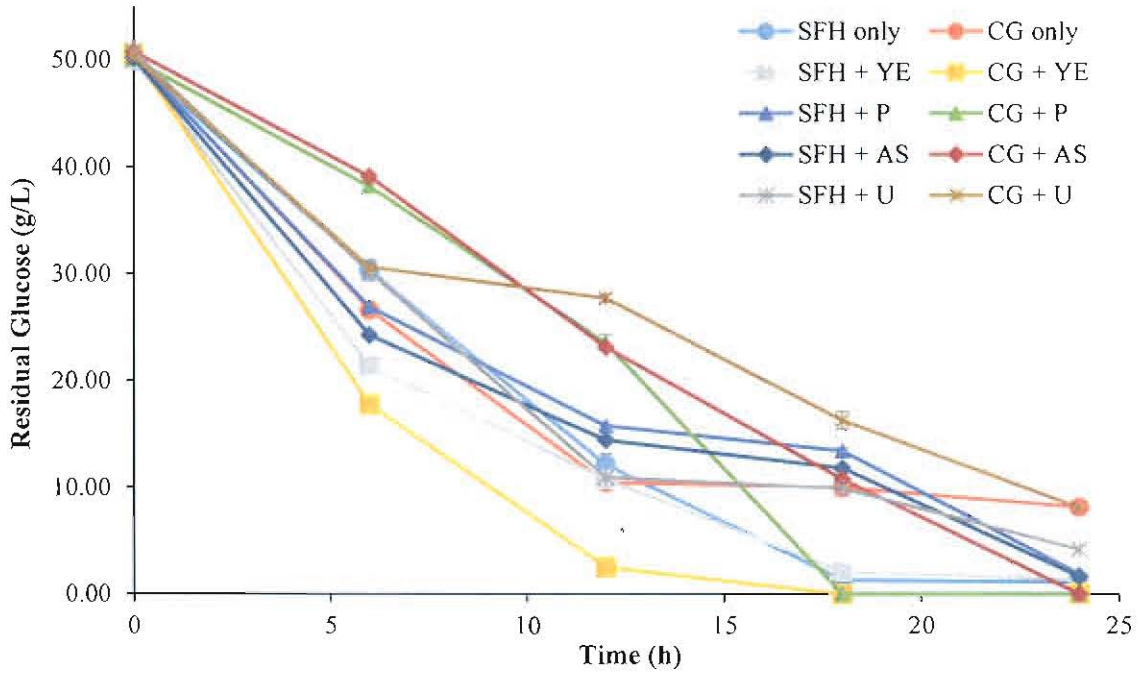


Figure 32: Effects of various nitrogen sources on glucose consumption profile during ethanol fermentation utilizing 50 g/L of glucose in SFH and CG media

This contradicts to (Tomas- Pejo *et al.*, 2012) which claimed that hydrolysate from lignocellulosics materials are low in nutrients and nitrogen. Not all lignocellulosic materials are low in nutrients, depends on it composition of the lignocellulosic fibres and other constituent. Besides, in CG media, it was observed that, glucose had been completely consumed by CBY during 18 hours of fermentation in media amended with YE and P only. In media amended with AS, glucose was fully consumed at 24 hours of fermentation. However, glucose was not fully utilized by the CBY during 24 hours of fermentation in media amended with Urea and CG media (without nitrogen source), with remaining 8.00 ±

0.44 g/L and 8.10 ± 0.10 g/L of glucose, respectively. This condition indicates that, the glucose consumption rate might be affected by the type of nitrogen source.

Laopaiboon *et al.* (2008) also reported on the same justifications, indicating the sugar remaining amount was affected by the amendment of nitrogen sources, because not all sugars were fully consumed by the yeast. This might be due to thermal stress as mentioned by Jones and Ingledew (1994). They discovered that the amount of sugar that could be fermented decreased when the fermentation temperature was above 25 °C. However, in this study, all fermentation was set at temperature 30 °C, thus glucose would be reduced significantly according to its different nitrogen sources. Apart from that, amino acids catabolism in the yeast cell probably would be activated, when yeast was starved for nitrogen source, due to the condition that yeasts would only consume low molecular weight of inorganic nitrogenous materials such as peptides, ammonium ions, amino acids, as well as urea (Russell, 2003).

4.6.2 Effects of Various Nitrogen Sources on Ethanol Production Profile

Figure 33 indicates the ethanol production profiles in 50 g/L of glucose from SFH and CG fermentation by CBY. It was observed that, in SFH media, ethanol was obtained in maximum amount faster during 18 hours of fermentation, and yet for 24 hours. In media containing peptone (P), maximum ethanol was produced (24.02 ± 0.01 g/L), followed by YE (22.71 ± 0.31 g/L) and control SFH media (20.33 ± 0.15 g/L), respectively. However, in media containing urea (U), maximum ethanol was achieved (20.13 ± 0.07 g/L) during 18 hours of fermentation, and less ethanol was produced in media containing ammonium sulfate (AS), which was 19.31 ± 0.02 g/L. The type of nitrogen sources does affect the ethanol formation, and yet improved the rate of fermentation.

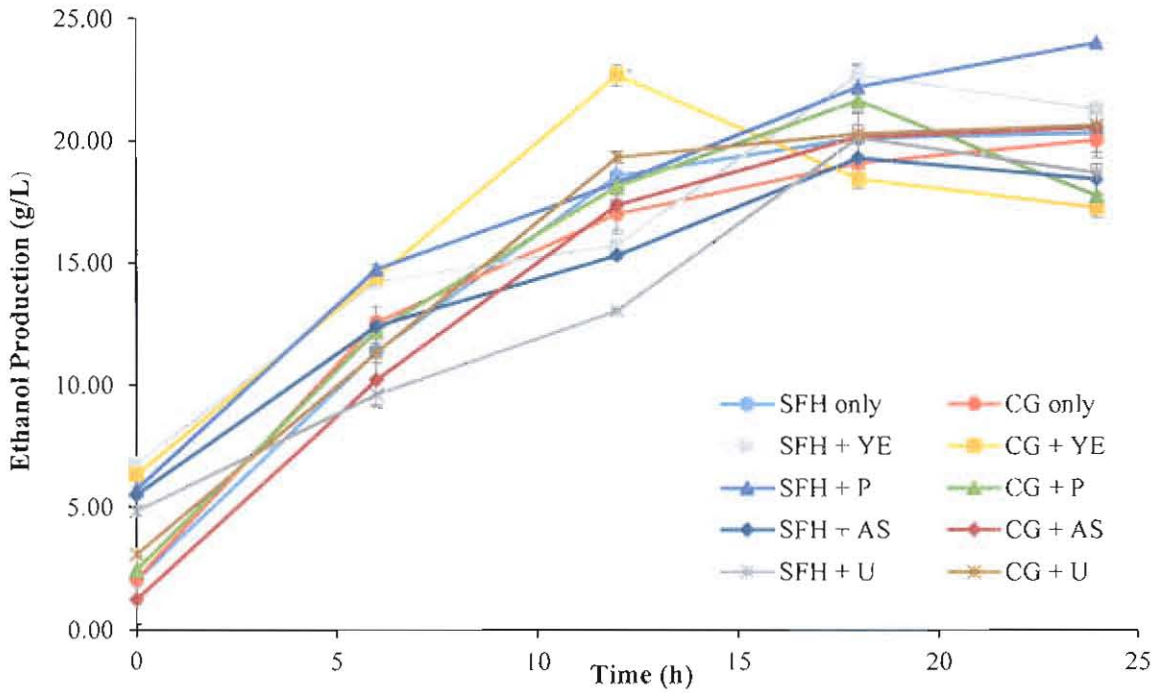


Figure 33: Effects of various nitrogen sources on ethanol production profile during ethanol fermentation utilizing 50 g/L of glucose in SFH and CG media

Compared with CG fermentation, ethanol achieved its maximum concentration faster during 12 hours of fermentation process, followed by 18 hours and lastly at 24 hours, in all type of nitrogen sources. It was about 22.70 ± 0.30 g/L of ethanol produced in fermentation utilizing YE, whereas in Peptone (P) media was 21.65 ± 0. 22 g/L, followed by Ammonium Sulfate (AS), urea (U) and CG control media, which was about 20.55 ± 0.54 g/L, 20.65 ± 0.05 g/L and 20.04 ± 0.04 g/L of ethanol being produced, respectively. There is no much differences on the amount of ethanol generated by CG media when amended with different nitrogen sources, except in media amended with YE and P, highest ethanol was produced.

According to this study, clearly, the type of nitrogen sources does affect the formation of ethanol in all different nitrogen sources, whether it enhanced the ethanol yield, thus increased the yeast growth or not. Previously, yeast extract (YE) was claimed as a complex nutrient, widely used as source of nitrogen for the yeast growth as well as nutrient supplement for ethanol production (Khongsay *et al.*, 2012). Due to expensive price, YE still can be replaced with other low – cost nitrogen sources such as peptone, ammonium sulfate and urea. Hence, the ethanol formation would be enhanced.

4.6.3 Effects of Various Nitrogen Sources on CBY Growth Profile (DCW)

Based on the CBY growth profile (Figure 34), it was observed that in SFH media containing YE, higher yeast growth was observed for the first 6 hours, and yet achieved maximum cell growth for 24 hours (7.50 ± 0.50 g/L) compared to other nitrogen sources. However, in media containing peptone (P), ammonium sulfate (AS) and control SFH were; (5.25 ± 0.75 g/L), (4.38 ± 0.13 g/L) and (4.75 ± 0.25 g/L), respectively. In this study, the CBY growth remained constant until the end of fermentation process, and yet the CBY does not enter death phase.

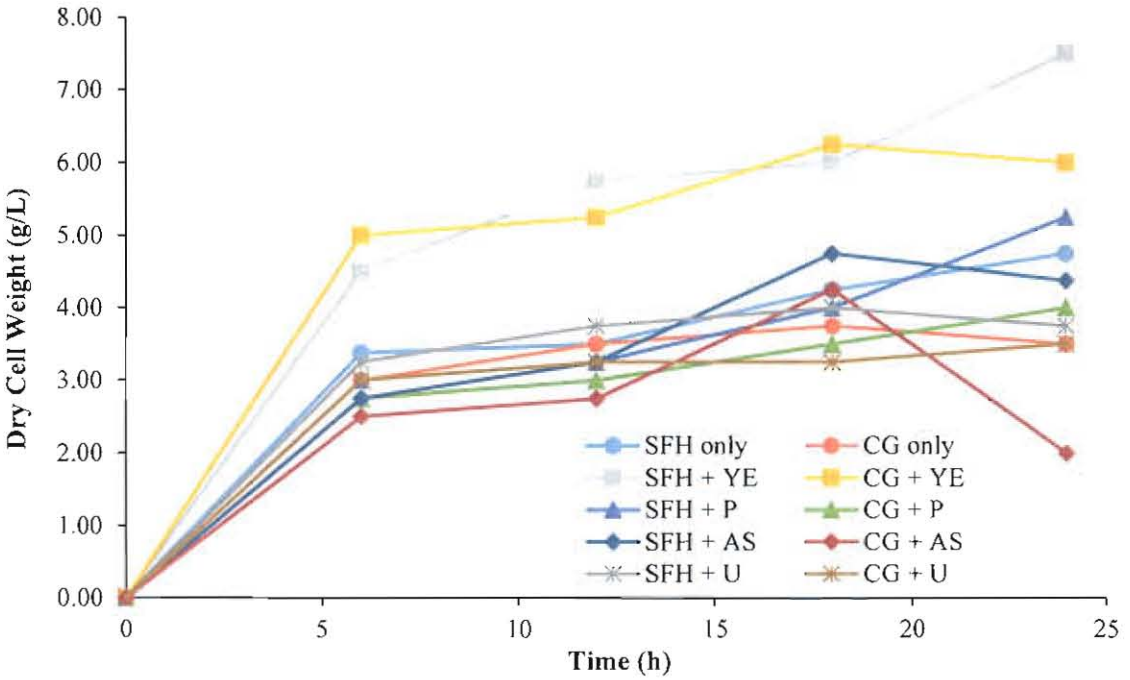


Figure 34: Effects of various nitrogen sources on CBY growth profile (DCW) during fermentation utilizing 50 g/L of glucose in SFH and CG media

According to Sauer and Mattanovich (2016), under caloric restriction or nutrient limitation without malnutrition, baker's yeast cells would dramatically reduce or constant in their growth rate due to the absence of carbon source, as well as essential amino acids. Thus, CBY cell would not enter death phase, as the glucose has been totally consumed and ethanol was produced in maximum. However, not all carbon sources would portray the same effects, as the exposure of yeast cells to survive under nutrient limitation was different according to type of yeast cells.

In CG fermentation, similar pattern of CBY growth was observed in media containing YE, which was higher growth, for the first 6 hours, and yet achieved maximum cell growth for 18 hours (6.25 ± 0.25 g/L) compared to other nitrogen sources. Although in peptone (P) and urea (U) supplemented, slightly slower growth of CBY was observed during the first 6 hour and 12 hours of fermentation process. However, in media containing AS, the yeast growth

was declined after 18 hours indicates that, the CBY was entering the death phase. In CG control media, the yeast shows constant growth rate until the end of fermentation process. The synthesis of cell biomass is clearly influenced by the nitrogen content in the growth medium, since the proportion of nitrogenous compounds in yeast cells is about 50% by weight. In addition, Pogaku and Sarbatly (2013) also claimed, with the addition of nitrogen source such as yeast extract and peptone, which are rich in amino acids could help the yeast to grow faster and produce more ethanol.

4.7 Sago Fibre Hydrolysate (SFH) as an Alternative Substrate for Production of Bioethanol by CBY: Verification Experiments using Statistical Analyses

Initially, the ethanol fermentation utilizing SFH itself without adding any nitrogen sources, has been conducted, and SFH portray very good performance for CBY to grow and generate alcohol. Maximum ethanol concentration was achieved in 50 g/L of glucose in SFH, thus contribute to high ethanol yield which was 0.42 ± 0.003 g/g. Residual glucose was almost metabolized by the CBY in fermentation broth due to the efficiency of fermentation process, as well as the assimilation of nutrients inside SFH media. However, this finding indicated that SFH should be amended with sufficient amount of nitrogen source for CBY to grow, thus improved the ethanol yield. This contradicts to studies done by Bernard *et al.* (2011), which define lignocellulosic substrate as poor sources of nutrients.

In this study, it was proven that SFH with 50 g/L of initial glucose, better yield of ethanol was achieved, eventhough without being supplemented with any nitrogen source. In addition to the carbon source, nitrogen source is very important factors for promoting the aerobic growth of commercial baker's yeast (CBY), and thus ethanol productivity. Table 14 depicts the kinetic parameters of ethanol fermentation in 50 g/L glucose from SFH by various types

of nitrogen supplements. This result suggested comparable ethanol yield (YE: 0.47 g/g, P: 0.50 g/g, AS: 0.50 g/g and 0.49 g/g) were detected for all nitrogen sources. It was observed that, ethanol was obtained maximum in different incubation period; which were faster at 18 hours of fermentation; Yeast Extract, Ammonium Sulfate and Urea, whereas in SFH media amended with Peptone and SFH (without addition of nitrogen source), ethanol was maximum during 24 hours of fermentation.

Table 14: Fermentation performance of batch ethanol fermentation utilizing 50 g/L glucose from SFH under various nitrogen supplements

Kinetic Parameters	**Nitrogen Sources**				
	Control (24 hrs)	**YE** (18 hrs)	**Peptone** (24 hrs)	**A. Sulfate** (18 hours)	**Urea** (18 hrs)
Initial glucose (g/L)	50.00 ± 0.00	50.00 ± 0.00	50.22 ± 0.21	50.15 ± 0.15	50.76 ± 0.04
Residual glucose (g/L)	1.11 ± 0.01	1.33 ± 0.03	1.86 ± 0.02	1.64 ± 0.03	4.18 ± 0.06
Maximum ethanol concentration (g/L)	20.33 ± 0.15	22.71 ± 0.31	24.02 ± 0.01	19.31 ± 0.02	20.13 ± 0.07
$Y_{p/s}$ (g/g)	0.42^{c} ± 0.003	0.47^{b} ± 0.005	0.50^{a} ± 0.002	0.50^{a} ± 0.002	0.49^{a} ± 0.002
E_f (%)	81.35^{c} ± 0.572	92.77^{b} ± 1.061	97.16^{a} ± 0.402	98.58^{a} ± 0.449	96.34^{a} ± 0.406
Q_p (g/Lh)	0.85^{d} ± 0.006	1.26^{a} ± 0.017	1.00^{c} ± 0.000	1.07^{b} ± 0.001	1.12^{b} ± 0.004

Notes: Means with the same letter are not significant at $p < 0.05$. Means that do not share a letter are significantly different at $\alpha = 0.05$.

From Table 14, it was revealed that ammonium sulfate (AS) was the best nitrogen source which give the highest ethanol yield (0.50^{a} ± 0.002 g/g). Fermentation efficiency in converting glucose to ethanol was highest in SFH (98.58^{a} ± 0.449%) when amended with AS. However, SFH media amended with Peptone (P) also depicted similar ethanol yield

($0.50^a \pm 0.002$ g/g), with fermentation efficiency of $97.16^a \pm 0.402\%$. SFH media amended with Urea (U) shows second highest ethanol yield which was at $0.49^a \pm 0.002$ g/g, with efficiency of its fermentation of $96.34^a \pm 0.406\%$. Moreover, based on this kinetic parameter, ethanol volumetric productivity for YE ($1.26^a \pm 0.017$ g/Lh) was much higher than U and AS, also indicates higher productivity ($1.12^b \pm 0.004$ g/Lh) and ($1.07^b \pm 0.001$g/Lh), compared to other nitrogen sources. Overall performances were significantly tested at significant level at $\alpha = 0.05$. Ethanol fermentation utilizing SFH as the carbon source which supplemented with ammonium sulfate (AS) as nitrogen source shows higher achievement compared to others nitrogen source. Referring to Arrizon and Gschaedler (2006), the most efficient nitrogen supplements for tequila fermentation was ammonium sulfate, as well as amino acids. Yeast extract, peptone and urea containing amino acids and peptides in their composition, thus able to improve ethanol yield and ethanol productivity. In common laboratory scale, yeast extract (YE) and peptone (P) are the most widely used as nitrogen sources. However, due to high cost, they are not possible for routine use in the industrial manufacture of a low profit margin ethanol (Chan- u-tit *et al.*, 2013).

Commonly in any fermentation industries, nitrogen sources are typically most expensive components of microbial growth media (Herpandi *et al.*, 2011). Several investigators also reported that yeast extract and peptone show more positive results and better as nitrogen supplements compared to urea and ammonium sulfate. But, the results are differed according to their specific substrate in their specific fermentation system. The addition of 1.4 g/L ammonium sulfate is lower than that added in corn steep liquor which is 2.3 g/L urea (Pereira *et al.*, 2010) but slightly higher when being supplemented in tequila fermentation, which is 1.0 g/L ammonium sulfate. Arrizon and Gschaedler (2006) revealed that different level of nitrogen concentration was required for different sources of raw materials used.

Furthermore, although the statistical analysis on kinetic parameters does not portray SFH control media as the best media, nonetheless, high ethanol still can be generated without addition of any nitrogen sources, which is more economical, suggesting SFH as low-cost fermentation media for the next and future generation. From this study, economical aspect of the fermentation process could trigger the production of ethanol from agricultural waste materials at large-scale based on the cheaper nutrient sources such as sago fibre. In overall, supplementation of cheaper nitrogen source such as ammonium sulfate (AS) and urea (U) together with SFH helps to minimize overall costing of ethanol production. However, all the kinetic parameters including the ethanol yield, ethanol volumetric productivity and fermentation efficiency must be at satisfactory value when considering to industrial level applications. Thus, sago fibre has very high potential to be promoted and commercially marketed as substrate in generating alcohols.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

Upon completion of this project, it has been revealed that glucose (92%) was the highest monomeric sugar component in sago fibre hydrolysate (SFH), obtained via enzymatic hydrolysis of sago fibre powder (SFP), discover high potential to be an alternative substrate for producing bioethanol using commercial baker's yeast. Analyses conducted shows that the enzymatic hydrolysis process utilizing Liquozyme SC DS and Spirizyme Fuel HS was effective enough in liberating the trapped starch, within the sago fibre into glucose. By employing the recycle method, higher glucose concentrations were obtained at the end of three- cycles of hydrolysis process.

Commercial baker's yeast (CBY) was selected to acclimatize for 9 hours which indicates the logarithmic phase of the growth profile and yet ready to be inoculated and transferred in all fermentation media. In this study, CBY was proved to continue grow steadily and even faster when inoculated into SFH compared to CG media. In all shake-flasks fermentation, CBY showed good performance towards the hydrolysate of sago fibre in all trials of 50 g/L and 80 g/L of initial glucose concentrations. Primarily, 50 g/L of glucose in both SFH and CG media shows highest glucose consumption efficiency, which was 97. 78% and 83.81%. SFH itself without adding any nitrogen sources shows good performance for CBY to grow and producing ethanol, thus contribute to high ethanol yield which was 0.42 ± 0.003 g/g. According to this study, 50 g/L of initial glucose concentration shows better ethanol fermentability compared to 80 g/L of initial glucose concentration. Among all the nitrogen sources that have been studied, 50 g/L of SFH amended with YE shows better enhancement towards the ethanol fermentability. However, YE claimed to be costly and should be replaced with other low-cost nitrogen sources. Based on statistical analysis towards kinetic

parameters of overall ethanol fermentation performance, it has been revealed that SFH amended with ammonium sulfate (AS) was the best nitrogen supplements in generating highest ethanol yield ($0.50^{a} \pm 0.002$ g/g), highest fermentation efficiency ($98.58^{a} \pm 0.449\%$) and higher ethanol volumetric productivity ($1.07^{b} \pm 0.001$ g/Lh), compared to peptone (P) and Urea (U). Overall ethanol fermentation performance indicates that SFH was comparable against CG because SFH portrays many great advantages, which ultimately improved the ethanol fermentability towards commercial baker's yeast (CBY), with respect to the yield, efficiency and productivity.

Interestingly, sago fibre hydrolysate (SFH) was discovered to be a highly potential substrate for ethanol production with or without the addition of ammonium sulfate (AS) as the nitrogen source. None of the metal ionic elements should be added in SFH due to existing nutrients and trace elements that enhanced the fermentation process. Therefore, by choosing commercial baker's yeast (CBY) as the superior microorganism to transform glucose into bioethanol is another highlighted part in this study, since baker's yeast is the least expensive microbes used for any industrial fermentation processes.

In the future research, it is recommended on the utilization of all part of lignocellulosic components within sago fibre (SF) including hemicellulose, cellulose and lignin. This is important for generating highest concentration of glucose, instead of using residual starch only. In order to obtain more glucose in SFH, the efficiency of the pre-treatment methods should be evaluated wisely to break down the cellulose and hemicellulose part successfully. Moreover, exploiting fresh sago fibre (SF) at the sago processing mills would be more economical rather than using dried sago fibre (SF) in producing glucose for subsequent hydrolysis and fermentation process. Thus, sago wastes can be managed properly at the sago mills instead of being discharged into nearby rivers or deposited at other places.

Apart from that, higher titre of bioethanol can be generated, by employing the very high gravity (VHG) ethanol fermentation of sago fibre (SF). Current enhancement in fermentation industries requires selection of others inoculum such as engineered yeast strains which able to digest both hexose and pentose sugars, simultaneously. Rather than generating bioethanol in the form of fermentation broth, purification on the pure ethanol should be initiated and thereafter, studies on antimicrobial properties should be conducted on both commercial ethanol and ethanol produced from SFH in various pharmaceutical sectors and medical sectors.

Conclusively, this study can be a reference for future study of sago fibre application into a novel application that will benefit local community especially sago planters and sago researchers meanwhile maintaining the sustainability of sago palm and its generation as the major commercial commodity, specifically in Sarawak.

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APPENDICES

Appendix A: Media/Reagents/Solution

Reducing Sugar Reagent (Miller, 1959)

Reagent:

1 g DNS 3, 5 acids

0.2 g Phenol

0.5 g Sodium Sulphite

1 g Sodium Hydroxide (NaOH)

Diluted to 100 mL distilled water

Rochelle Salt: Dilute 40 g Potassium Sodium Tartarate in 100 mL distilled water

Starch Colorimetric using Iodine Method (Nakamura, 1981)

Reagent:

Iodine (0.01M)

Potassium Iodide, KI (0.25M)

A volume of 70 mL KI was dissolved in an approximately 0.01 mL amount of iodine. The volume was brought up to 100 mL and kept in dark reagent bottle.

Potato Dextrose Agar (PDA)

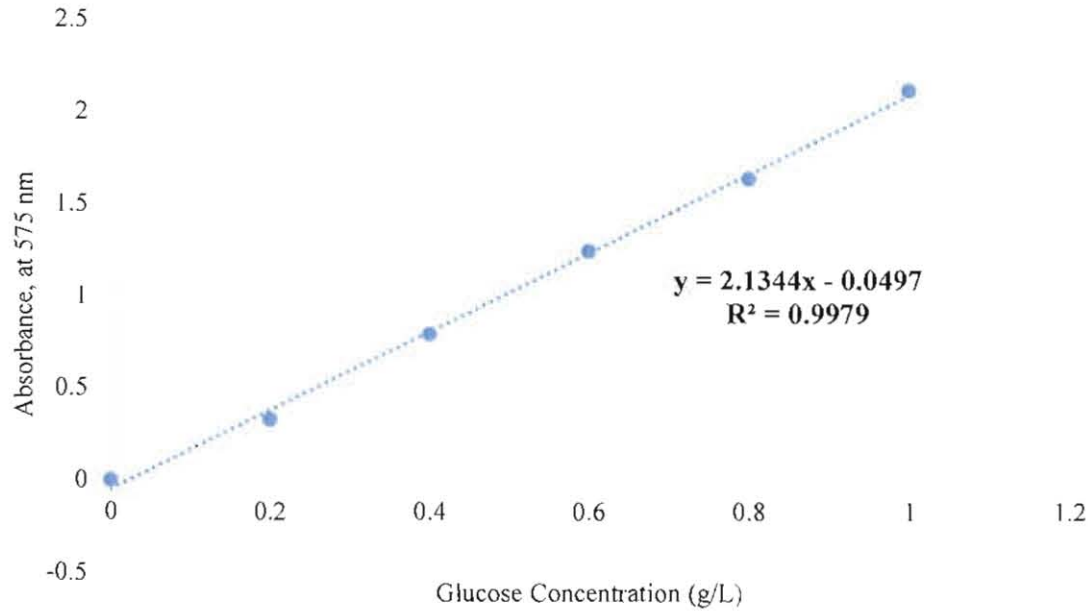
39.0 g Bacto^{TM} PDA powder, dissolved in 1000 mL distilled water.

Yeast Malt Broth (YMB) Medium

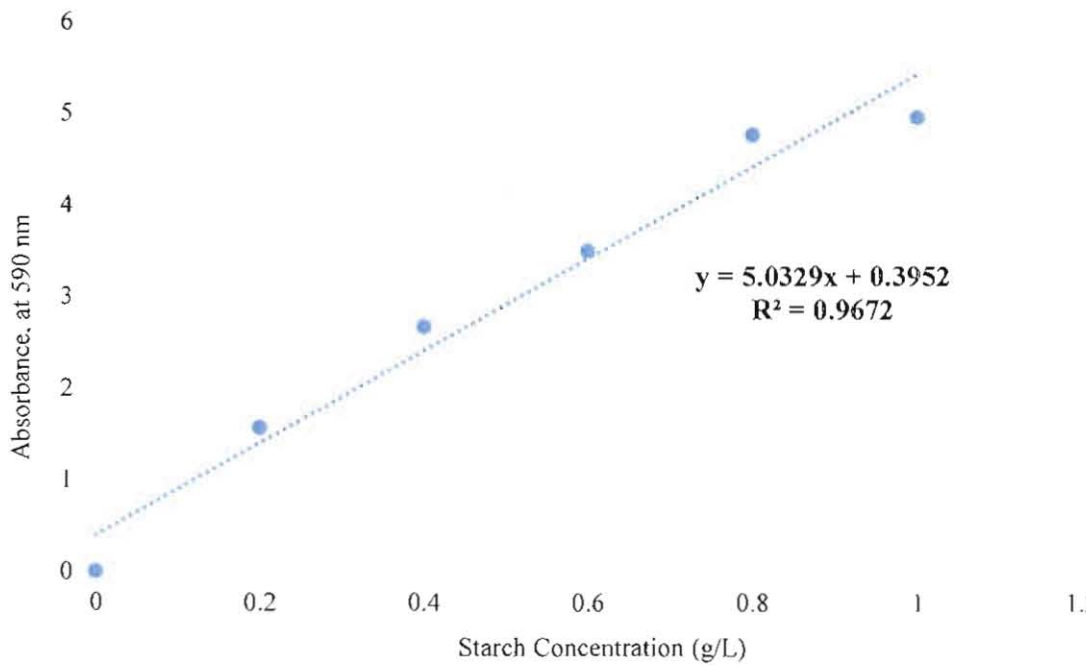
1.05 g of Sigma-Aldrich YMB powder, dissolved in 50 mL distilled water.

Appendix B: Standard Calibration Curves

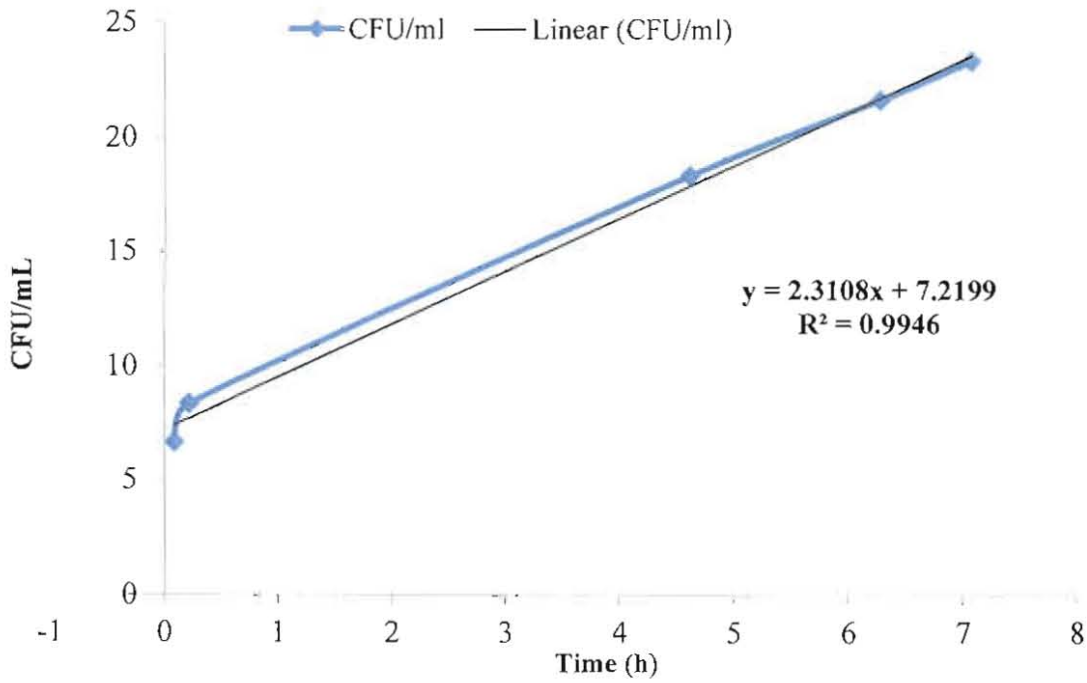
Standard Calibration Graph of Glucose Concentration



Standard Calibration Graph of Starch Concentration



Standard Calibration Graph of Commercial Baker's Yeast (CBY)



Appendix C: Tabulated Data and Statistical Data

Appendix C1: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L SFH control medium.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.00 ± 0.00	2.03 ± 0.03	0.00 ± 0.00
6	30.30 ± 2.23	11. 37 ± 0.20	3.38 ± 0.13
12	12.13 ± 0.48	18.60 ± 0.31	3.50 ± 0.00
18	1.33 ± 0.08	20.13 ± 0.05	4.25 ± 0.25
24	1.11 ± 0.01	20.33 ± 0.15	4.75 ± 0.25

Appendix C2: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L CG control medium.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.28 ± 0.10	2.07 ± 0.02	0.00 ± 0.00
6	26.62 ± 0.29	12.58 ± 0.48	3.00 ± 0.00
12	10.47 ± 0.19	17.02 ± 0.56	3.50 ± 0.00
18	9.99 ± 0.01	19.10 ± 0.10	3.75 ± 0.25
24	8.14 ± 0.44	20.04 ± 0.04	3.50 ± 0.00

Appendix C3: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L SFH with YE supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.00 ± 0.00	6.78 ± 0.17	0.00 ± 0.00
6	21.41 ± 0.30	14.24 ± 0.09	4.50 ± 0.00
12	10.77 ± 0.17	15.76 ± 0.19	5.75 ± 0.25
18	2.10 ± 0.10	22.71 ± 0.31	6.00 ± 0.00
24	1.33 ± 0.03	21.31 ± 0.11	7.50 ± 0.50

Appendix C4: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L SFH with Peptone (P) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.22 ± 0.21	5.74 ± 0.18	0.00 ± 0.00
6	26.87 ± 0.17	14.74 ± 0.14	3.00 ± 0.00
12	15.79 ± 0.09	18.29 ± 0.33	3.25 ± 0.25
18	13.42 ± 0.22	22.21 ± 0.21	4.00 ± 0.00
24	1.86 ± 0.02	24.02 ± 0.01	5.25 ± 0.75

Appendix C5: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L SFH with Urea (U) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.76 ± 0.04	4.84 ± 0.14	0.00 ± 0.00
6	30.14 ± 0.08	9.63 ± 0.36	3.25 ± 0.25
12	10.94 ± 0.16	13.05 ± 0.05	3.75 ± 0.25
18	9.88 ± 0.06	20.13 ± 0.07	4.00 ± 0.00
24	4.18 ± 0.06	18.72 ± 0.04	3.75 ± 0.75

Appendix C6: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L CG with YE supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.53 ± 0.02	6.36 ± 0.36	0.00 ± 0.00
6	17.77 ± 0.23	14.43 ± 0.14	5.00 ± 0.00
12	2.51 ± 0.49	22.70 ± 0.30	5.25 ± 0.75
18	0.00 ± 0.00	18.46 ± 0.30	6.25 ± 0.25
24	0.00 ± 0.00	17.29 ± 0.29	6.00 ± 0.00

Appendix C7: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L CG with Peptone (P) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.02 ± 0.02	2.44 ± 0.04	0.00 ± 0.00
6	38.16 ± 0.16	12.24 ± 0.24	2.75 ± 0.25
12	23.53 ± 0.53	18.15 ± 0.15	3.00 ± 0.00
18	0.00 ± 0.00	21.65 ± 0.22	3.50 ± 0.00
24	0.00 ± 0.00	17.78 ± 0.08	4.00 ± 0.50

Appendix C8: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L CG with Ammonium Sulfate (AS) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.76 ± 0.14	1.23 ± 0.10	0.00 ± 0.00
6	39.14 ± 0.27	10. 24 ± 0.09	2.50 ± 0.00
12	23.13 ± 0.20	17.39 ± 0.39	2.75 ± 0.25
18	10.75 ± 0.15	20.17 ± 0.13	4.25 ± 0.25
24	0.00 ± 0.00	20.55 ± 0.54	2.00 ± 0.00

Appendix C9: Average of residual glucose, ethanol and dry cell weight concentrations for 50 g/L CG with Urea (U) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	50.30 ± 0.05	3.07 ± 0.06	0.00 ± 0.00
6	30.59 ± 0.25	11.33 ± 0.29	3.00 ± 0.00
12	27.73 ± 0.28	19.34 ± 0.15	3.25 ± 0.25
18	16.30 ± 0.60	20.30 ± 0.26	3.25 ± 0.25
24	8.10 ± 0.10	20.65 ± 0.05	3.50 ± 0.00

Appendix C10: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L SFH control medium.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	80.06 ± 0.04	2.57 ± 0.03	0.00 ± 0.00
12	64.37 ± 1.12	7.63 ± 0.27	4.50 ± 0.19
24	53.72 ± 1.32	12.22 ± 0.50	5.05 ± 0.05
36	38.50 ± 1.95	12.33 ± 0.32	6.01 ± 0.02
48	24.16 ± 1.40	13.32 ± 0.09	4.84 ± 0.04

Appendix C11: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L CG control medium.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	79.98 ± 0.08	1.85 ± 0.13	0.00 ± 0.00
12	71.72 ± 0.68	5.16 ± 0.47	5.08 ± 0.01
24	61.46 ± 0.82	11.44 ± 0.54	4.53 ± 0.06
36	51.20 ± 1.04	12.19 ± 0.13	4.51 ± 0.02
48	48.99 ± 0.99	12.98 ± 0.03	0.55 ± 0.07

Appendix C12: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L SFH with YE supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	80.54 ± 0.44	2.10 ± 0.00	0.00 ± 0.00
12	58.66 ± 4.23	22.82 ± 0.57	5.28 ± 0.20
24	9.93 ± 0.04	40.66 ± 0.12	5.51 ± 0.00
36	0.00 ± 0.00	41.04 ± 0.04	7.61 ± 0.23
48	0.00 ± 0.00	40.41 ± 0.25	4.84 ± 0.04

Appendix C13: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L SFH with Peptone (P) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	79.16 ± 0.04	2.62 ± 0.00	0.00 ± 0.00
12	55.03 ± 0.28	17.30 ± 1.05	4.70 ± 0.36
24	42.12 ± 0.99	22.54 ± 1.08	1.46 ± 0.10
36	17.14 ± 5.37	31.77 ± 0.67	0.51 ± 0.15
48	4.15 ± 1.69	35.75 ± 0.13	0.07 ± 0.01

Appendix C14: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L SFH with Ammonium Sulfate (AS) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	79.32 ± 0.12	2.60 ± 0.03	0.00 ± 0.00
12	65.96 ± 3.24	14.59 ± 1.17	4.94 ± 0.32
24	6.25 ± 0.24	38.54 ± 1.38	7.51 ± 0.12
36	0.00 ± 0.00	37.46 ± 0.45	6.09 ± 0.03
48	0.00 ± 0.00	40.05 ± 0.04	5.10 ± 0.07

Appendix C15: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L SFH with Urea (U) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	80.01 ± 0.00	3.00 ± 0.10	0.00 ± 0.00
12	34.40 ± 1.31	17.63 ± 0.37	7.74 ± 1.18
24	6.09 ± 0.54	26.89 ± 2.24	4.72 ± 0.25
36	0.00 ± 0.00	31.78 ± 0.46	6.15 ± 0.03
48	0.00 ± 0.00	36.39 ± 0.49	4.61 ± 0.01

Appendix C16: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L CG with YE supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	80.51 ± 0.41	2.35 ± 0.29	0.00 ± 0.00
12	51.94 ± 1.02	18.33 ± 0.31	5.53 ± 0.26
24	27.93 ± 0.31	25.19 ± 0.33	5.59 ± 0.30
36	13.49 ± 0.42	33.96 ± 0.04	6.20 ± 0.02
48	2.15 ± 0.09	33.07 ± 0.03	7.27 ± 0.18

Appendix C17: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L CG with Peptone (P) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	79.98 ± 0.08	1.43 ± 0.29	0.00 ± 0.00
12	61.64 ± 0.64	17.98 ± 0.35	4.47 ± 0.21
24	42.36 ± 0.96	23.41 ± 0.59	5.23 ± 0.09
36	19.40 ± 1.34	28.23 ± 0.01	5.01 ± 0.02
48	6.55 ± 0.03	31.10 ± 0.05	2.79 ± 0.06

Appendix C18: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L CG with Ammonium Sulfate (AS) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	79.53 ± 0.51	2.31 ± 0.33	0.00 ± 0.00
12	59.42 ± 0.78	18.01 ± 0.86	4.71 ± 0.01
24	39.63 ± 0.40	26.00 ± 0.40	4.91 ± 0.01
36	18.93 ± 0.31	31.53 ± 0.48	3.60 ± 0.25
48	4.41 ± 0.22	35.37 ± 0.33	2.52 ± 0.43

Appendix C19: Average of residual glucose, ethanol and dry cell weight concentrations for 80 g/L CG with Urea (U) supplement.

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)	Biomass Concentration (g/L)
0	79.45 ± 0.55	2.38 ± 0.34	0.00 ± 0.00
12	41.13 ± 1.07	13.67 ± 0.51	3.49 ± 0.30
24	20.82 ± 0.73	22.14 ± 0.61	4.41 ± 0.37
36	15.35 ± 1.09	30.62 ± 0.08	4.52 ± 0.00
48	1.54 ± 0.52	33.48 ± 0.16	5.03 ± 0.01

Enzymatic Hydrolysis of Residual Starch in Sago Fibre (SF) for Production of Glucose

Appendix C20: One- way ANOVA and Tukey tests on the glucose production of three-cycle enzymatic hydrolysis of SF.

Sample	Mean	StDev	F-value	P-value
Cycle III	149.36[a]	2.66	490. 75	0.000
Cycle II	100.10[b]	5.13		
Cycle I	46.24[c]	3.93		

*Notes: Means that do not share a letter are significantly different at $\alpha = 0.05$.

Comparison of Kinetic Parameters in Ethanol Fermentation utilizing 50 g/L glucose of SFH media under Various Nitrogen Supplements.

a) Ethanol Yield, $Y_{p/s}$

Appendix C21: One-way ANOVA for ethanol yield ($Y_{p/s}$) under various nitrogen supplements.

Sample	Mean	StDev	F-value	P-value
SFH + AS	0.50[a]	0.003	123.14	0.000
SFH + P	0.50[a]	0.003		
SFH + U	0.49[a]	0.003		
SFH + YE	0.47[b]	0.008		
SFH only	0.42[c]	0.004		

*Notes: Means that do not share a letter are significantly different at $\alpha = 0.05$.

b) Fermentation Efficiency, E_f(%)

Appendix C22: One- way ANOVA for fermentation efficiency (%) under various nitrogen supplements.

Sample	Mean	StDev	F-value	P-value
SFH + AS	98.58[a]	0.635	123.14	0.000
SFH + P	97.16[a]	0.569		
SFH + U	96.34[a]	0.574		
SFH + YE	92.77[b]	1.500		
SFH only	81.35[c]	0.809		

*Notes: Means that do not share a letter are significantly different at $\alpha = 0.05$.

c) Ethanol Volumetric Productivity, Q_p

Appendix C23: One-way ANOVA for ethanol volumetric productivity (Q_p) under various nitrogen supplements.

Sample	Mean	StDev	F-value	P-value
SFH + YE	1.26[a]	0.024	336.22	0.000
SFH + U	1.12[b]	0.006		
SFH + AS	1.07[b]	0.002		
SFH + P	1.00[c]	0.001		
SFH only	0.85[d]	0.009		

*Notes: Means that do not share a letter are significantly different at $\alpha = 0.05$.

(Department of Statistics, 2015)

6.3 EKSPORT HASIL RUMBIA 2004 – 2013
Export of Sago Products 2004 – 2013

Tahun *Year*	Tepung & Bijian Rumbia *Sago Flour and Meal*		Kanji Rumbia *Sago Starch*		Empelur Rumbia *Sago Pith*		Jumlah *Total*	
	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM
2004	4,104.90	3,066,119	42,899.03	33,953,479	0.00	0	47,003.93	37,019,598
2005	198.00	183,150	45,332.43	40,432,542	0.00	0	45,530.43	40,615,692
2006	0.00	0	42,870.90	42,955,166	0.00	0	42,870.90	42,955,166
2007	0.00	0	44,766.88	51,407,110	0.00	0	44,766.88	51,407,110
2008	792.00	686,381	44,424.75	57,067,424	0.00	0	45,216.75	57,753,805
2009	2,034.00	1,788,352	41,484.64	60,403,459	0.00	0	43,518.64	62,191,811
2010	0.00	0	44,448.83	62,831,429	1.50	1,065	44,450.33	62,832,494
2011	0.00	0	50,726.78	90,949,230	0.00	0	50,726.78	90,949,230
2012	162.00	145,265	47,687.26	86,199,739	0.00	0	47,849.26	86,345,004
2013 (p)	**0.00**	**0**	**47,946.37**	**80,978,108**	**0.00**	**0**	**47,946.37**	**80,978,108**

(p) Permulaan

(p) Preliminary

Sumber Jabatan Perangkaan

Source Department of Statistics

6.4 IMPORT HASIL RUMBIA 2004 – 2013
Import of Sago Products 2004 – 2013

Tahun *Year*	Tepung & Bijian Rumbia *Sago Flour and Meal*		Kanji Rumbia *Sago Starch*		Empelur Rumbia *Sago Pith*		Jumlah *Total*	
	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM
2004	0.00	0	10.25	13,390	31.50	43,499	41.75	56,889
2005	38.29	66,990	0.00	0	19.92	34,060	58.21	101,050
2006	0.00	0	67.57	107,378	32.22	63,808	99.79	171,186
2007	0.00	0	53.44	109,306	13.53	27,315	66.97	136,621
2008	0.00	0	151.72	261,196	15.59	34,948	167.31	296,144
2009	0.00	0	77.41	136,867	29.85	59,292	107.26	196,159
2010	8.25	20,000	126.58	233,407	34.69	72,940	169.52	326,347
2011	26.55	92,642	75.97	203,304	0.48	2,520	103.00	298,466
2012	11.05	46,956	120.31	330,527	1.02	6,044	132.38	383,527
2013 (p)	**10.25**	**21,020**	**69.14**	**197,243**	**5.25**	**13,680**	**84.64**	**231,943**

(p) Permulaan

(p) Preliminary

Sumber Jabatan Perangkaan

Source Department of Statistics

6.5 EKSPORT TEPUNG DAN BIJIAN RUMBIA MENGIKUT NEGERI DITUJUI 2009 – 2013

Export of Sago Flour and Meal by Country of Destination 2009 – 2013

Negeri *Country*	2009		2010		2011		2012		2013(p)	
	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM
Japan	2,034.00	1,788,352	0.00	0	0.00	0	162.00	145,265	0.00	0
Jumlah *Total*	**2,034.00**	**1,788,352**	**0.00**	**0**	**0.00**	**0**	**162.00**	**145,265**	**0.00**	**0**

(p) : Permulaan

(p) : *Preliminary*

Sumber : Jabatan Perangkaan

Source : Department of Statistics

6.6 EKSPORT KANJI RUMBIA MENGIKUT NEGERI DITUJUI 2009 – 2013

Export of Sago Starch by Country of Destination 2009 – 2013

Negeri *Country*	2009		2010		2011		2012		2013(p)	
	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM	tan metrik *tonne*	RM
China, Peoples Rep of	66.00	101,200	66.00	114,444	44.00	118,853	24.00	70,853	22.00	4,840
Japan	11,804.00	18,390,093	13,997.09	19,210,036	12,985.81	25,616,887	13,002.00	25,355,592	12,398.00	21,807,553
Korea, Rep of	0.00	0	0.00	0	122.00	271,550	40.00	98,000	0.00	0
Malaysia (Peninsular)	23,775.56	33,801,205	24,209.57	34,406,070	32,383.25	52,991,509	29,559.51	49,784,777	30,927.15	49,583,530
Malaysia (Sabah)	244.50	333,225	277.50	299,288	430.00	430,000	100.03	143,600	268.64	428,065
Singapore, Rep of	3,731.40	4,618,962	2,834.13	4,191,317	2,977.00	6,633,937	2,844.00	6,114,490	2,602.00	5,405,176
Taiwan	121.00	165,958	720.00	958,420	100.00	147,000	100.00	132,000	0.00	0
Thailand	1,196.00	1,933,648	1,670.21	2,425,475	1,282.00	3,764,748	954.00	2,164,457	998.08	2,099,579
United States of America	18.18	26,361	90.90	129,987	72.72	156,348	72.72	191,980	0.00	0
Vietnam, Socialist Rep of	526.00	1,032,808	583.44	1,096,392	330.00	818,400	858.00	1,902,472	686.00	1,475,640
Myanmar, Union of	0.00	0	0.00	0	0.00	0	45.00	47,887	22.50	25,137
Philippines	0.00	0	0.00	0	0.00	0	88.00	195,831	22.00	49,588
Jumlah *Total*	**41,484.64**	**60,403,459**	**44,448.84**	**62,831,429**	**50,726.78**	**90,949,230**	**47,687.26**	**86,189,739**	**47,946.37**	**80,878,108**

(p) : Permulaan

(p) : *Preliminary*

Sumber : Jabatan Perangkaan

Source : Department of Statistics

Proximate Analysis of Sago Fibre Powder (SFP)

MAKMAL DIAGNOSA VETERINAR NEGERI SARAWAK

Batu Satu,Jalan Datuk Mohd .Musa 93250 Kota Samarahan | Peti Surat 1395 93728 Kuching | Tel: 082 - 612949 Telegram: VETLAB

LAPORAN MAKMAL

Rujukan Tuan: VDL D.I. No: 193/15(f)

Tarikh: Tarikh: 25.03.2015

Kepada:
Fakulti FSTS,
UNIMAS,
Kota Samarahan,
Sarawak.

sk. Pengarah Pertanian Sarawak
U.P: ADV (H)

U.P: Encik Zakaria B. Ismail

Tarikh dihantar: 25.02.2015 Tarikh diterima: 26.02.2015

Tuanpunya dan Alamat: Fakulti FSTS, UNIMAS, Kota Samarahan, Sarawak.

Sago Waste from Sago Mill Industries Malaysia

Keputusan Makmal:

No	Jenis Contoh, Kandungan (%)	Hampas Sagu % secara di terima
1	Kelembapan Moisture content	10.43
2	Protein Kasar Crude Protein	3.56
3	Gentian Kasar Crude Fibre	22.94
4	Lemak Kasar Total Crude Fat	0.13
5	Jumlah Abu Total Ash	3.35
6	Tenaga Kasar (kalori/g) Energy	3957.63

Catatan:

Surat Asal Ditandatangani
Oleh
Dr. Andrea Lim Li Li

Tandatangan : .....
Nama : (DR. ANDREA LIM LI LI)
Pegawai Penguasa Veterinar
Makmal Diagnosa Veterinar Negeri
Kota Samarahan, Sarawak.

Laporan ini tidak boleh digunakan untuk percetakan tanpa kebenaran dari Pegawai Veterinar Penguasa Berkenaan

Nutrient/ Trace Elements Analysis in Sago Fibre Hydrolysate (SFH)

MAKMAL DIAGNOSA VETERINAR NEGERI SARAWAK

Batu Satu,Jalan Datuk Mohd Musa 93250 Kota Samarahan | Peti Surat 1395 93728 Kuching | Tel 082 - 612949 Taligram VETLAB

LAPORAN MAKMAL

Rujukan Tuan. VDL D.I. No: 1532/15(f)

Tarikh: Tarikh: 18.04.2016

Kepada:
Universiti Malaysia Sarawak,
UNIMAS,
94300 Kota Samarahan,
Kuching.

sk. Pengarah Pertanian Sarawak
U.P: ADV (H)

U.P: Sharifah Bt Mohammad

Tarikh dihantar: 16.12 2015 Tarikh diterima: 18.12.2015

Tuanpunya dan Alamat: Sharifah Bt Mohammad. Universiti Malaysia Sarawak, UNIMAS, 94300 Kota Samarahan, Kuching.

Keputusan Makmal:

No	Jenis Contoh	Hampas Sagu A	Hampas Sagu B
	Kandungan	mg/ L secara di terima	mg/ L secara di terima
1	Phosphorus	3663.8	4916.3
2	Potassium	2840.9	2460 9
3	Calcium	101.17	305 33
4	Magnesium	46.78	118.81
5	Iron	0.70	2.78
6	Manganese	3.51	8.58
7	Copper	0.03	0.04
8	Zinc	0.36	0.91
9	Boron	0 45	0.43

Catatan: Hantar ke ARC pada 30/12/2015, keputusan diterima pada 18/04/2016.

Tandatangan : .....
Nama : (MOHAMMAD BIN BRAHIM)
b.p. Pegawai Penguasa Veterinar
Makmal Diagnosa Veterinar Negeri
Kota Samarahan, Sarawak.

Laporan ini tidak boleh digunakan untuk percetakan tanpa kebenaran dari Pegawai Veterinar Penguasa Berkenaan

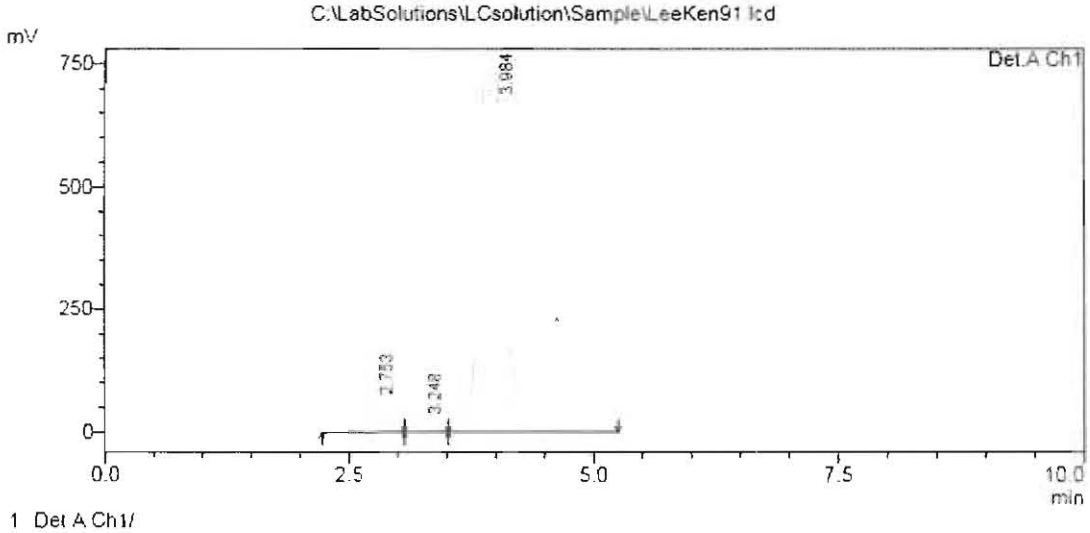
HPLC Chromatogram of Sugar Component in Sago Fibre Hydrolysate (SFH)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\LCsolution\Sample\LeeKen91.lcd

Acquired by	: Admin
Sample Name	: PAH SHH 100g/L
Sample ID	: bob testing
Vail #	:
Injection Volume	: 20 uL
Data File Name	: LeeKen91.lcd
Method File Name	: PDA_Method.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 8/18/2016 3:56:08 PM
Data Processed	: 8/18/2016 4:06:09 PM

<Chromatogram>



1 Det.A Ch1/

PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.753	927759	64617	6.180	7.788
2	3.248	382335	25587	2.547	3.084
3	3.984	13702772	739445	91.274	89.128
Total		15012866	829648	100.000	100.000

Retention time:

(1) 2.753: Oligosaccharides

(2) 3.248: Cellobiose

(3) 3.984: Glucose

Appendix D: Conferences and Expo

No.	Conference	Date	Venue	Participation
1.	12th Asian Congress on Biotechnology (ACB) 2015	15-19th November 2015	Istana Hotel, Kuala Lumpur	Poster presentation Title: Supplementation of Commercial Nitrogen Sources on Hydrolysate of Sago Hampas for Bioethanol Production
2.	3rd ASEAN Sago Symposium (ASAS) 2016	25-26th October 2016	CAIS, UNIMAS	Oral Presentation Title: Sugars from Residual Starch of Sago Fibre: Source of Food and Energy
3.	Innovation Technology Expo (InTex) 2017	17-18th May 2017	Detar Putra, UNIMAS	Poster Presentation Title: Sagostar: The Golden Syrup (Silver Award) Title: E-Fibrosa: Eco-friendly Bioethanol from Sago Fibre Hydrolysate (SFH) (Bronze Award)