



Faculty of Resource Science and Technology

Production of Liquid Biofuel from Fresh Sago Effluent

Huang Chai Hung

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Production of Liquid Biofuel from Fresh Sago Effluent

Huang Chai Hung

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DECLARATION

I hereby declare that the dissertation entitled, ‘Production of Liquid Biofuel from Fresh Sago Effluent’, is based on my original work except for quotations and citations which have duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

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Huang Chai Hung

Matric No.: 15020195

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ABSTRACT

Sago starch processing factories are generally located nearby waterways, where wastewater is normally discharged, resulting in environmental issues. Sago effluent comprises mainly of macromolecules in the form of polysaccharides that can potentially be exploited to produce bioethanol. Hence, in this study, sago effluent and its constituents were used for bioethanol production in addition to recover the energy lost in the waste stream as well as purifying the effluent. Prior to the enzymatic saccharification and fermentation process, the sago effluent was subjected to starch and fibre analysis using phenol-sulphuric acid assay, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF) and Klason lignin determination as well as determination of ash content using standard protocol. Based on the result of compositional analysis, total sago effluent (TSE) used was comprised $97.00 \pm 0.02\%$ sago effluent hydrolysate and $3.00 \pm 0.02\%$ sago hampas. After further compositional analyse, the sago effluent hydrolysate was found to be consisting of $2.00 \pm 0.02\%$ total carbohydrate, while sago hampas was composed of $55.40 \pm 0.02\%$ starch, $23.64 \pm 0.77\%$ cellulose, $9.07 \pm 1.18\%$ hemicellulose, $4.01 \pm 0.51\%$ lignin, and $2.23 \pm 0.01\%$ ash. The initial stage of the study was utilised sago effluent hydrolysate for bioethanol production via simultaneous saccharification and fermentation (SSF) with the aid of amylolytic enzymes and the yeast *Saccharomyces cerevisiae*. Results indicated that sago effluent hydrolysate was efficient in generating high ethanol yield at 8.39 ± 0.20 g/l ($84.89 \pm 4.56\%$ Theoretical Ethanol Yield; TEY) as its production was insignificant different compared to commercial starch loading (8.88 ± 0.07 g/l or $91.49 \pm 0.74\%$ TEY). Next, SSF was conducted on sago hampas at 2.5%, 5.0% and 7.5% (w/v) feedstock loadings for 5-day with aid of amylolytic enzymes, commercial cellulase and fermentative synthesis of ethanol of *S. cerevisiae*, to

establish the optimum feedstock loading for ethanol production. The highest ethanol production was detected in 5.0% (w/v) sago hampas at 17.79 ± 0.17 g/l or $79.65 \pm 0.78\%$ TEY at 48 h. This was significantly higher than ethanol productions in the broth with 2.5% and 7.5% (w/v) sago hampas loadings, which were 8.38 ± 0.07 g/l ($75.00 \pm 0.63\%$ TEY) and 23.28 ± 0.61 g/l ($69.48 \pm 1.81\%$ TEY) respectively. However, by corresponding to TEY, 7.5% (w/v) sago hampas loading was shown lowest ethanol production due to the inhibition of high carbohydrate content on cellulase activities. The final study in this research the optimisation of the simultaneous saccharification and bioethanol fermentation process, termed as sequential saccharification and simultaneous fermentation (SSSF). SSSF was conducted on TSE by involving amylolytic stage and cellulolytic stage in order to minimise the inhibition of high sugar content on cellulase activities. High carbohydrate and glucose concentrations were detected at 0 h, indicating the carbohydrate-rich of TSE and the initial saccharification of starch by amylases added at $50\text{ }^{\circ}\text{C}$. Additionally, occurrence of glucose extinction was due to the efficient conversion by the yeast. Meanwhile, the slightly increased of total carbohydrate was marking the starting of cellulolytic stage. The highest ethanol production was peaked at 20.01 ± 0.73 g/l, corresponding to $86.56 \pm 3.17\%$ TEY at 48 h. At the end of fermentation period, 3.50 ± 0.20 g/l of residual carbohydrates was detected as there was no utilisation of xylose and arabinose by the yeast. As a conclusion, sago effluent and its constituents were found to be potentially excellent feedstocks for bioethanol production via saccharification and fermentation process.

Keywords: Bioethanol; sago effluent; simultaneous saccharification and fermentation (SSF); sequential saccharification and simultaneous fermentation (SSSF); *Saccharomyces cerevisiae*.

Penghasilan Biofuel Cecair daripada Efluen Sagu Segar

ABSTRAK

Kilang pemprosesan kanji sagu biasanya terletak berdekatan dengan sungai, di mana air sisa akan dialir keluar menyebabkan isu-isu alam sekitar. Efluen sagu mengandungi makromolekul dalam bentuk polisakarida yang berpotensi untuk dieksploitasi dalam penghasilan bioetanol. Oleh itu, efluen sagu telah digunakan dalam kajian ini untuk menghasilkan bioetanol, di samping memulihkan tenaga serta penapisan efluen. Sebelum proses hidrolisis enzim dan penapaian, efluen sagu telah melalui analisis kanji dan serat dengan menggunakan kajian asid fenol-sulfuric, Serat Detergen Asid (ADF), Serat Detergen Neutral (NDF) dan kajian klason lignin serta penentuan kandungan abu melalui protokol piawai. Hasil kajian menunjukkan bahawa efluen sagu terdiri daripada $97.00 \pm 0.02\%$ sagu sisa cecair dan $3.00 \pm 0.02\%$ sisa pepejal sagu. Berdasarkan analisis, hidrolisat efluen sagu mengandungi $2.00 \pm 0.02\%$ karbohidrat, manakala hampas sagu terdiri daripada $55.40 \pm 0.02\%$ kanji, $23.64 \pm 0.77\%$ selulosa, $9.07 \pm 1.18\%$ hemiselulosa, $4.01 \pm 0.51\%$ lignin dan $2.23 \pm 0.01\%$ abu. Pada mulanya, hidrolisat efluen sagu digunakan untuk menghasilkan bioetanol melalui sakrafikasi dan fermentasi serentak (SSF) dengan bantuan enzim amilolitik dan yis Saccharomyces cerevisiae. Daripada kajian ini, hidrolisat efluen sagu mampu menjana etanol yang tinggi pada 8.39 ± 0.20 g/l (84.89% hasil etanol teori) dan tidak berbeza berbanding dengan penghasilan daripada kanji komersial. Oleh itu, hidrolisat efluen sagu mempunyai potensi sebagai bahan mentah menghasilkan bioetanol. Seterusnya, SSF telah dijalankan ke atas 2.5%, 5.0% and 7.5% (w/v) hampas sagu selama 5 hari dengan bantuan enzim amilolitik, selulase komersial dan sintesis penapaian etanol, S. cerevisiae,

untuk mengenalpasti kepekatan bahan mentah yang optimum dalam penghasilan etanol. Penghasilan etanol tertinggi adalah 5.0% (w/v) hampas sagu, iaitu 17.79 ± 0.17 g/l atau $79.65 \pm 0.78\%$ TEY pada jam ke-48. Keputusan ini jauh lebih tinggi daripada penghasilan etanol daripada 2.5% and 7.5% (w/v) hampas sagu, 8.38 ± 0.07 g/l ($75.00 \pm 0.63\%$ TEY) dan 23.28 ± 0.61 g/l ($69.48 \pm 1.81\%$ TEY) masing-masingnya. Berdasarkan TEY, 7.5% (w/v) hampas sagu telah menunjukkan penghasilan etanol yang paling rendah kerana perencatan kandungan karbohidrat yang tinggi ke atas aktiviti selulase. Bahagian akhir kajian ini adalah mengoptimumkan proses hidrolisis dan fermentasi bioetanol melalui proses sakrafikasi berturut dan fermentasi serentak (SSSF). SSSF telah dijalankan ke atas efluen sagu untuk mengurangkan perencatan kandungan gula yang tinggi kepada aktiviti selulase. Kandungan karbohidrat dan glukosa yang tinggi telah dikesan pada permulaan, menunjukkan kekayaan karbohidrat dalam efluen sagu dan sakrafikasi awal kanji oleh amilase yang ditambah pada 50 °C. Selain itu, kehabisan glukosa berlaku disebabkan oleh kecekapan penukaran oleh yis. Sementara itu, sedikit peningkatan dalam jumlah karbohidrat telah menandakan permulaan peringkat selulolitik. Penghasilan etanol tertinggi memuncak pada 20.01 ± 0.73 g/l bersamaan dengan $86.56 \pm 3.17\%$ TEY pada jam ke-48. Pada akhir tempoh penapaian, 3.50 ± 0.20 g/l karbohidrat sisa dikesan kerana tiada penukaran xylosa dan arabinosa oleh yis. Kesimpulannya, effluent sagu dan kandungannya dikenalpasti mampu dijadikan sebagai bahan mentah bagi penghasilan bioetanol melalui sakrafikasi dan fermentasi proses.

Kata Kunci: Bioetanol; efluen sagu; sakrafikasi dan fermentasi serentak (SSF); sakrafikasi berturut dan fermentasi serentak (SSSF); Saccharomyces cerevisiae.

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LIST OF ABBREVIATIONS

ADF	Acid Detergent Fibre
AFEX	Ammonia fiber expansion
ANOVA	One-way analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bio-ETBE	Bio-ethyl tertiary butyl ether
BOD	Biological oxygen demand
C ₂ H ₅ OH	Ethanol
(C ₅ H ₈ O ₄) _n	Hemicellulose
(C ₆ H ₁₀ O ₅) _n	Starch
(C ₆ H ₁₀ O ₅) _n	Cellulose
C ₆ H ₁₂ O ₆	Glucose
CH ₃ -CH ₂ OH	Ethanol
CH ₃ CH ₂ OH	Ethanol
CO ₂	Carbon dioxide
COD	Chemical oxygen demand
Da	Dalton
DP	Degree of polymerisation
EC	Energy Commission
EtOH	Ethanol
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester

FPU/g	Filter Paper Unit per gram
g/l	gram per liter
g/mol	gram per mole
h	hour
H ₂ O	water
H ₂ SO ₄	sulphuric acid
HPLC	High Performance Liquid Chromatography
Kcal/kg	kilocalorie per kilogram
kDa	kilodalton
kg/m ³	kilogram per cubic meter
kJ/kg	kilojoule per kilogram
kJ/L	kilojoule per liter
M	Molarity
min	minute
MJ/kg	megajoule per kilogram
ml	millilitre
ml/min	millilitre per minute
mm	millimetre
mM	milliMolarity
mmHg	millimetre of mercury
mPa.s	millipascal-second
MSW	Municipal solid wastes
NADH	Nicotinamide adenine dinucleotide
NDF	Neutral Detergent Fibre

NExBTL	Neste Renewable Diesel
nm	nanometre
O ₂	oxygen
OD	Optical Density
OH	Oxygen-Hydrogen
pH	Potential of Hydrogen
pK _a	-log ₁₀ of acid dissociation constant
PPO	Pure plant oil
PSA	Phenol-sulphuric acid
p-value	probability value
R ²	R-squared
REE	Rape ethyl ester
RFA	Renewable Fuels Association
RME	Rape methyl-ester
rpm	revolutions per minute
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SD	Standard deviation
SHF	Separate hydrolysis and fermentation
SNG	Synthetic natural gas
SSF	Simultaneous saccharification and fermentation
SSSF	Sequential saccharification and simultaneous fermentation
TCA	Tricarboxylic acid
TEY	theoretical ethanol yield
Tons	tonnes

μl	microliter
μm	micrometre
U/ml	Unit per millilitre
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
wt	weight
x g	Relative centrifugal force
YM	Yeast Malt
YP-CB	Yeast peptone citrate buffer

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

With the current increasing in energy demands, supply shortage is one of the biggest challenge that humans have to tackle with in this new millennium (Baeyens *et al.*, 2015). Essentially, energy is not only a strategic national resource but also plays a central role from individual welfare to social and industrial developments (Ji & Long, 2016). Until now, energy needs have been met primarily by the employ of fossil fuels (natural gas, oil and coal), as the source for heat and electrical energy production (Ibrahiem, 2015; Ji & Long, 2016; Patel, 2014). These fossil feedstocks are also used for cooking and the production of many essential products such as transportation fuel, pharmaceuticals, detergents, fine chemicals, synthetic fibre, plastics, solvents and lubricants (Adekunle *et al.*, 2016; Naik *et al.*, 2010).

The combustion of petroleum-based fuels is one of the major contributors to the net emission of greenhouse gases that leads to global warming (Ben-Iwo *et al.*, 2016). The overuse of fossil fuels has also negatively impact human health due to the release of air pollutants such as carbon monoxide and nitrogen oxides. These air pollutants can cause headache, cardiac diseases and respiratory infections (Patel, 2014). Additionally, oil prices are very volatile and after World War II, the price of oil and other fossil fuels has affected the expansion and contraction of world economy (Ibeto *et al.*, 2011; Patel, 2014). The over-dependence on fossil fuels is depicted as an obstacle for the sustainable economic development because the

supply of the fossil feedstocks is finite and non-renewable. Therefore, these petroleum-based products are not environmentally, ecologically and economically sustainable. In response, these have spawned increased public and scientific attentions in the quest for alternative energy sources, particularly biofuel (Gupta & Verma, 2015; Shah & Sen, 2011).

Biofuels are predominantly produced from renewable feedstocks and can exist as solid, liquid or gaseous fuels (Ben-Iwo *et al.*, 2016; Tye *et al.*, 2011). Biofuel is commonly used as liquid transportation fuel because of its renewability, sustainability, availability, cleanliness and economic efficiency. Furthermore, the use of biofuel for transportation can solve environmental problems by partly or completely replacing gasoline, contributing to a significant reduction in the harmful greenhouse gases emissions (Balat, 2011; Daylan & Ciliz, 2016; Ji & Long, 2016).

Liquid biofuel such as bioethanol, is the most vital as an alternative energy source to substitute petroleum-based transportation fuels in the future (Gupta & Verma, 2015; Karmee, 2016). Ethanol has a high heat of vaporisation and high octane but low cetane number that impede self-ignition in a diesel engine. These spectacular properties enable bioethanol to be developed into the suitable material for the mixed gasoline fuel in a spark-ignition engine (Aditiya *et al.*, 2016; Chin & H'ng, 2013; Mansouri *et al.*, 2016). Besides its vehicular application, bioethanol has the potential to provide uses in pharmaceutical, cosmetic, beverage, medical and other industrial applications. In Malaysia, bioethanol can contribute to the practical benefits in the economic and social sectors if implemented nationally (Aditiya *et al.*, 2016; Chin & H'ng, 2013).

Traditionally, via first-generation platform, bioethanol is produced from sucrose-containing feedstocks and starchy sources (Bhatia *et al.*, 2012; Ritslaid *et al.*, 2010; Shah & Sen, 2011). For instance, the United States and Brazil are the largest biofuel producers that started mass production of bioethanol from corn and sugarcane, respectively (Chin & H'ng, 2013; Ibeto *et al.*, 2011). Previously, the primary uses of these feedstocks have been for food and animal feed production. As a result, the production of biofuel using these raw materials has led to a conflict with the industry of food supply, and eventually increasing the demand on the food crops supply. The development of first-generation bioethanol became unattainable due to the concerns on food security (Daylan & Ciliz, 2016; Naik *et al.*, 2010). Subsequently, the inexpensive lignocellulosic biomass was put forward as a feasible alternative material for the second-generation bioethanol production (Anwar *et al.*, 2014; Limayem & Ricke, 2012).

Lignocellulosic biomass is mainly under-utilised waste. These are locally available bioresources that are abundant in nature, such as forestry wastes, food processing, municipal solid wastes, agricultural residues and other industrial wastes, as well as dedicated energy crops (Jensen & Orlygsson, 2012; Shah & Sen, 2011; Singh *et al.*, 2015). These non-edible lignocellulosic biomasses are made from a heterogeneous complex of carbohydrate polymers, which are cellulose, hemicellulose and lignin (Verma *et al.*, 2011). According to Prasad *et al.* (2007), different types of plant materials contain a variable proportion of each component. These biomasses represent an as-of-yet untapped fermentable sugars sources for significant industrial use, offering limited conflict with food crops, large-scale availability with a low cost and lower fossil fuel inputs (Jenol *et al.*, 2014; Zheng & Rehmann, 2014). Several literatures have noted that with advanced technologies, second-generation

bioethanol can be produced by releasing the sugars locked in polysaccharides and then fermented into ethanol by specific organisms (Adekunle, *et al.*, 2016; Behera *et al.*, 2014; Chin & H'ng, 2013).

In Malaysia, transportation is mainly operated on gasoline as compared to diesel as there are larger numbers of vehicles with spark-ignition engines compared to compression-ignition engines. With the significant amount of industrial activities, the abundant industrial wastes could be promising alternative sources for second-generation bioethanol production in Malaysia (Aditiya *et al.*, 2016). Hence, in this study, the production of bioethanol from fresh sago effluent and its constituents was attempted.

Sago effluent is a carbohydrate-rich wastewater that could be further utilised to produce valuable chemicals, particularly bioethanol. The utilisation of sago effluent could become a viable solution to the popular deteriorating fuel demand, as well as environmental complications such as pollution and waste management (Aditiya *et al.*, 2016; Sangeetha *et al.*, 2015). The bulk of the effluent is composed of 94%-97% liquid, while the remaining is solid waste (Awg-Adeni *et al.*, 2010). The initial stage of the current study involves utilising sago effluent hydrolysate for ethanol production via simultaneous saccharification and fermentation (SSF). During the sago starch extraction, large volume of water is used and sago effluent that contains residual starch is discarded into the waterways (Awg-Adeni *et al.*, 2010; Bujang, 2014). Hence, in the current study, the starch content in sago effluent hydrolysate was converted into fermentable sugars using amylases (Ritslaid *et al.*, 2010). *Saccharomyces cerevisiae* was employed as fermenting microorganism as it is efficient in

the production of ethanol and able to withstand a high concentration of ethanol (Piškur *et al.*, 2006; Reis *et al.*, 2013).

The solid waste portion of sago effluent, known as sago hampas, is a starchy lignocellulosic biomass that contains up to 58.0% starch and 26.0% cellulose concentrations (Jenol *et al.*, 2013; Vincent *et al.*, 2015b). Awg-Adeni *et al.* (2012) reported this utilisation of this biomass as a substrate for microbes to produce reducing sugar and enzyme, for hydrolysis into confectioner's syrup, animal feed, in particleboard manufacturing and compost for mushroom culture. Sago hampas is also currently being evaluated for its potential as a raw material in the production of ethanol via SSF with the aid of amylase, cellulase and yeast. According to Vincent *et al.* (2015a), previous studies only utilise either the starch portion or fibre residues for the production of bioethanol. Therefore, in the current study, SSF was employed to produce ethanol from both the starch portion and fibre residues of sago hampas without any pre-treatment. SSF was chosen for both raw materials as it is documented as being superior in the yield of ethanol (Sarkar *et al.*, 2012; Tomás-Pejó *et al.*, 2009).

The cellulase activities can be inhibited by the accumulation of monosaccharides and this then causes the reduction of glucose yields (Hsieh *et al.*, 2014). It is imperative to develop a new method that can improve the ethanol production by maximising the activities of cellulase in saccharifying the cellulose content. Therefore, to address this obstacle, sequential saccharification and simultaneous fermentation (SSSF) was conducted to determine the efficiency of total sago effluent for bioethanol production in the final part of the current study. SSSF is the modified version of SSF, a sequential process in which the starch portion was first liquefied and saccharified, followed by the hydrolysis of the fibre

residues. These stages were achieved by employing amylases and cellulases to break down complex polysaccharides into simple sugars prior to the fermentation process (Vincent *et al.*, 2015a).

1.2 Hypothesis

Many studies were carried out to solve the current predicament faced by the Malaysian sago industry due to its improper management. But, most of the previous studies only utilise either the starch portion or fibre residues of sago hampas for bioethanol production. The current studies were utilised not only sago hampas, but also sago effluent hydrolysate and total sago effluent. Thus, bioethanol can be produced from total sago effluent and its constituents without any pre-treatment throughout the saccharification and fermentation process.

1.3 Research Objectives

The main goal of the current study is to generate a biological process of fresh sago effluent conversion into fermentable sugars and followed by the production of ethanol, as a renewable and sustainable energy source.

Specifically, the studies were conducted to meet the following objectives:

- a. To evaluate the potential of sago effluent hydrolysate as a substrate for the bioethanol production.
- b. To investigate the optimum sago hampas loading for production of bioethanol.
- c. To perform sequential saccharification and simultaneous fermentation (SSSF) on total sago effluent for bioethanol production

1.3 Thesis Organisation

This thesis comprises of six chapters. An introduction on the importance of the research and the objectives of the study discussed in the first chapter. Chapter 2 provides a comprehensive literature review on the sago palm, sago starch, sago effluent and its constituents. This is followed by the discussion on starches and an introduction to lignocellulosic biomass with its components and contributions toward the production of ethanol. After that, the discussion flows into biorenewable energy, ethanol and bioethanol. The production of ethanol as fuel and Malaysia's perspective are also covered. Finally, the chapter concludes with discussions on the organism used in this study, *S. cerevisiae* and the types of amylolytic and cellulolytic enzymes involved in the simultaneous saccharification and fermentation (SSF) process.

The subsequent chapters consist three manuscripts:

Chapter 3 – “Bioethanol Production from Sago Effluent Hydrolysate using *Saccharomyces cerevisiae* via Simultaneous Saccharification and Fermentation (SSF)” presents the potential of sago effluent hydrolysate as a substrate for the bioethanol production via SSF using

amylases and *S. cerevisiae*. The chapter purposes to accomplish the first objective of this study.

Chapter 4 – “Production of Bioethanol from Sago Hampas via Simultaneous Saccharification and Fermentation (SSF)” documents the bioethanol production from sago hampas, via enzymatic hydrolysis and followed by of the fermentable sugars into ethanol using *S. cerevisiae*, through SSF. The second objective of the study is addressed in this chapter, which is to identify the optimum feedstock loadings for bioethanol production from sago hampas.

Chapter 5 – “Utilisation of Total Sago Effluent as Feedstock for Bioethanol Production via Sequential Saccharification and Simultaneous Fermentation (SSSF)” details the application of SSSF on total sago effluent via sequential liquefaction and saccharification of starch, followed by hydrolysis of fibre residue, with *S. cerevisiae* as the fermenting microorganism. A chapter was prepared to cover the last objective of this thesis.

Finally, Chapter 6 delivers the general conclusions and research recommendation. Tables and figures are inserted within the main texts of every chapter. All literature cited are listed at the end of this thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Sago Palm and Sago Starch

Sago palm (*Metroxylon sagu* Rottb.) belongs to a group of starch-bearing palms, is a species of the genus *Metroxylon*. The name *Metroxylon* is derived from the Greek words, ‘metra’ and ‘xylon’, meaning parenchyma and xylem respectively (Singhal *et al.*, 2008). The word ‘sago’ comes from Javanese, which meant starch-containing sago pith (Singhal *et al.*, 2008). It is of commercial importance as the main source of starch, as starch is considered as most important food sources (Hasyim *et al.*, 2011; Yunus *et al.*, 2014). Figure 2.1 shows the sago palm plantations in Sarawak, East Malaysia.



Figure 2.1: Sago palm plantation in Sarawak, East Malaysia (Singhal *et al.*, 2008).

An extremely hardy sago palm thrives in acidic, swampy, saline and submerged peat soils. This plant grows well in humid tropical lowland forest and can also be found in freshwater swamps. The sago palm grows well when relative air humidity of 70% and temperatures above 25 °C (Uthumporn *et al.*, 2014). The full-grown sago has stem that reaches up to 2000 cm long and the fruits clusters need take up to 24 months to mature. The stem of sago palm grown in clumps are very thick with a diameter of 40 cm and maximum height of 2500 cm, and the leaves are pinnate. This palm can produce both non-pollinated and pollinated fruits. The sago leaflets are about 150 cm in length and the spadix is 350 to 450 cm long with the spathes that are quite spinelss, called linear-ensiform leaflets (Awg-Adeni *et al.*, 2010). Sago palm is also categorised as hapaxantic and also grouped as soboliferous or suckering (Uthumporn *et al.*, 2014).

Sago diversity is centred in Papua New Guinea due to the presence of the high genetic variation and the vast natural stands of sago palm trees (Awg-Adeni *et al.*, 2012; Karim *et al.*, 2008). Unfortunately, sago-based industries there is established without any significant development. On the contrary, the industry is well established in Sarawak, East Malaysia and has become an important contributing industry to export revenue (Karim *et al.*, 2008; Naim *et al.*, 2016). Oya-Dalat, Igan, Mukah, Pusa-Saratok, and Balingan are major sago plantation areas in Sarawak. It is due to the suitability of the peat soil for sago cultivation (Karim *et al.*, 2008).

Sago palm promotes as a socially stable agroforestry system as it is economically acceptable, relatively sustainable, environmentally friendly, uniquely versatile and vigorous (Awg-Adeni *et al.*, 2010). Virtually all products that derived from sago palm have local uses. The

rachis and bark are most commonly used as building material and firewood in factories. The leaflets of sago are used as roofing material, woven into ropes, bags, baskets and cages, and utilised as food wrap as well as spoons. (Awg-Adeni *et al.*, 2010; Singhal *et al.*, 2008). In Malaysia, New Guinea, Indonesia and Philippines, sago palm is grown commercially for conversion to ethanol fuel and animal food of pigs, horses and chickens (Singhal *et al.*, 2008). However, sago starch obtained from the trunks of sago palm is the most important sago palm derived product (Yunus *et al.*, 2014; Singhal *et al.*, 2008).

Sago starch accumulation in the trunk begins from the base upwards and can reach above 250 kg per dry weight per plant (Nozaki *et al.*, 2004). According to Singhal *et al.* (2008), the positions of starch storage differ based on the conditions of soil that palms grow in. The sago palm grown in mineral soil has most starch accumulation at a height of 600 cm and below, with the higher amount at height 300 to 350 cm. Hence, starch is almost absence in between 850 to 920 cm. Conversely, in acidic soil, the maximum amount of the starch concentration of palm is located at top with a height of 700 to 780 cm (Nozaki *et al.*, 2004; Singhal *et al.*, 2008). Furthermore, the concentration of starch is higher in sago palms grown in mineral soil compared to the palms that grown acid sulfate soil (Nozaki *et al.*, 2004).

Sago starch is generally smooth-surfaced granule with an average diameter of 32 μm , smaller than those of potato, but bigger than those of cassava, rice, wheat and corn. The physicochemical of sago starch is shown in Table 2.1. The quality of sago starch produced is influenced by the inconsistency of sago logs. It is due to the physiological growth of the sago palm affects on the sago starch content at different growth stages (Pei-Lang *et al.*, 2006). Generally, sago palm growth can be classified into four stages, which are Plawei,

Bubul, Angau Muda and Angau Tua. The sago starch content is detected to rise as the palms matured from Plawei to Angau Muda, but then decline from Angau Tua to late Angau Tua stage. Hence, the highest starch content is found at Angau Muda stage, which are 41.3% and 41.4% at base and mid height respectively.

Table 2.1: Physicochemical composition of sago starch (Karim *et al.*, 2008; Singh *et al.*, 2008; Swinkels, 1985).

Granule shape	Oval
Granule size	10-50 μm
Amylose	27%
Amylopectin	73%
Lipids	0.10%
Protein (nitrogen content x 6.25)	0.10%
Ash	0.20%
Phosphorus	0.02%

Traditionally, sago starch extraction can be classified into two levels, which are domestic and small-scale processing plant levels (Karim *et al.*, 2008). Due to rapid industrialisation, new technologies with fully mechanical processes are acquired by the large-scale sago factories for starch extraction (Singhal *et al.*, 2008). Sago logs go through debarking, rasping, sieving, settling washing and drying for sago starch extraction. Further disintegration is performed on the chips obtained by the aid of hammer mill. Prior to further purification that conducted in a nozzle separator, coarse fibre is removed from the resulting starch slurry by passing through a series of centrifugal sieves until pure starches is obtained. The watering of the starch is through rotary drum, followed by hot air drying (Awg-Adeni *et al.*, 2010). Sago bark and sago effluent are discarded from sago mills into nearby water bodies during the process of starch extraction (Hasyim *et al.*, 2011; Vincent *et al.*, 2015a). The schematic flow diagram for sago starch extraction shown in figure below (Figure 2.2).

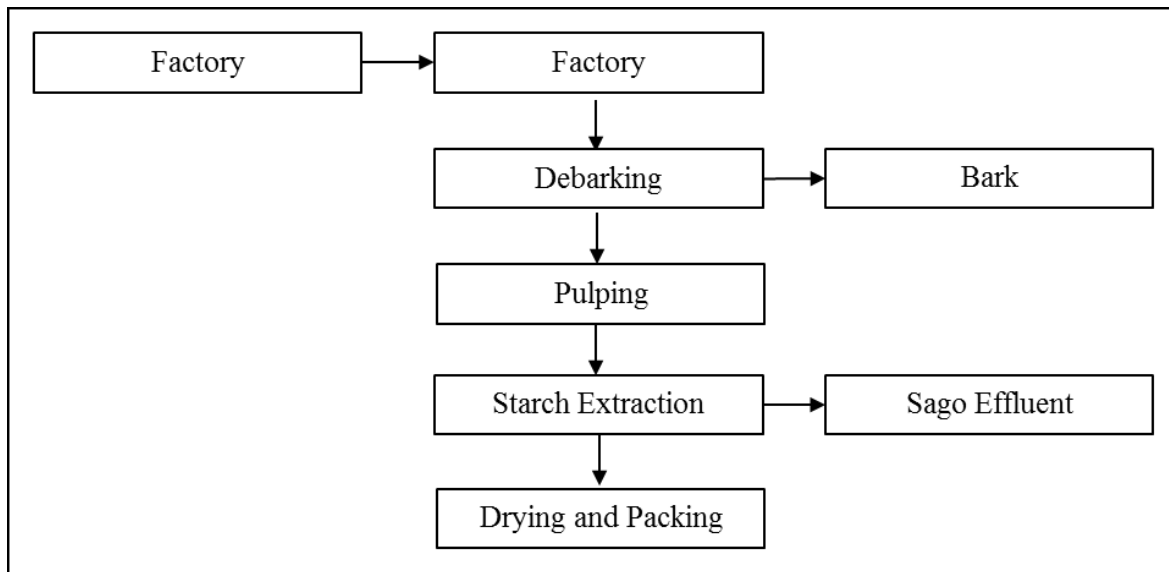


Figure 2.2: Schematic flow diagram for sago starch extraction (Awg-Adeni *et al.*, 2010).

2.2 Sago Effluent

In recent years, the ever-increasing sago starch demands are caused the increase in the volume of sago effluent disposed from sago mills (Bujang, 2014; Sangeetha *et al.*, 2015). According to Bujang (2014), the processing of 600 logs produced about 240 tons of sago effluents. It is estimated that approximately 10^6 litres of wastewater are produced for every 1 tonne of sago hampas generated from a single sago starch processing mill per day (Hasyim *et al.*, 2011). The bulk of this wastewater is composed of 94% to 97% of sago liquid waste, while the remaining (4.0% or 9.6 tons) is hampas (Awg-Adeni *et al.*, 2010; Bujang, 2014).

Sago effluent, carbohydrate-rich wastewater, is referred to as effluent due to its obnoxious odour, irritating colour, lower pH, high chemical oxygen demand (COD) and biological oxygen demand (BOD) (Ayyasamy *et al.*, 2002; Ruban *et al.*, 2013). Additionally, sago hampas consists a significant number of starch granule material (Figure 2.3). It is an outcome

of the inefficient mechanical process employed for sago starch extraction and often ceases to dislodge starch residues trapped within the fibrous portion of the trunk (Awg-Adeni *et al.*, 2012; Karim *et al.*, 2008). Additionally, cellulose is another abundant naturally occurring macromolecular material in sago hampas (Pushpamalar *et al.*, 2006). On dry basis, sago hampas contains up to 23.0% cellulose, 9.2% hemicellulose, 4.0% lignin and 58.0% starch (Awg-Adeni *et al.*, 2012; Thangavelu *et al.*, 2014). The wastewater is likely to be discharged directly into the waterways without proper treatment, which contravene the standard limit discharge enacted in the Environmental Quality Act, 1974. Hence, the untreated wastewaters result in intense contamination of the rivers due to its slow degradability characteristic in the environment (Awg-Adeni *et al.*, 2012; Yunus *et al.*, 2014).

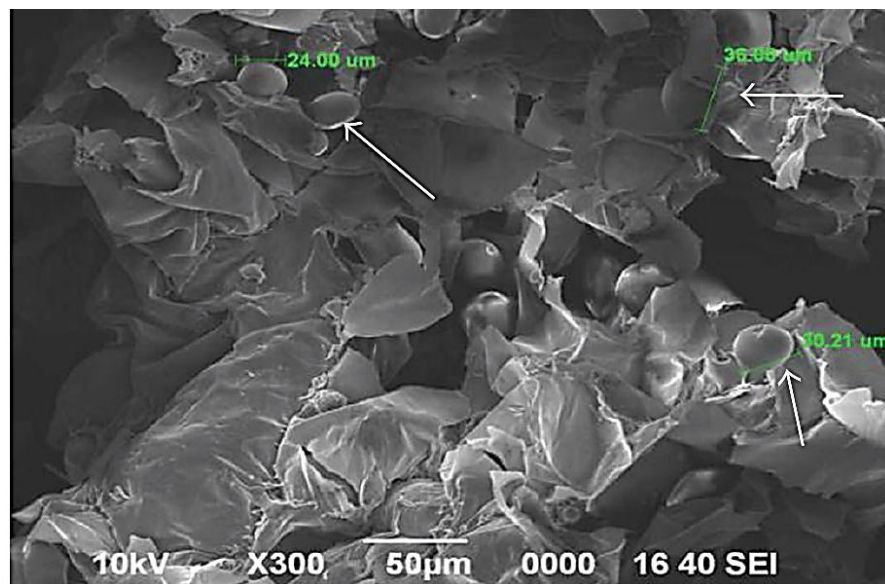


Figure 2.3: Starch granules (white arrow) were embedded within the sago hampas via SEM (Awg-Adeni *et al.*, 2012).

Many studies have been carried out to solve the current predicament faced by the Malaysian sago industry due to its improper management. Sago effluent is suitable for an up-flow packed bed digester under anaerobic fermentation as it consists a very high carbon to

nitrogen ratio (105:0.12). In addition, this wastewater also contains sufficient nutrients to support algal growth with an average carbon to nitrogen to phosphorus ratio (24:0.14:1). Thus, it is possible to harness the voluminous sago effluent as a cheap and profitable cultured medium in producing single cell protein (Bujang, 2014; Phang *et al.*, 2000). Another study also reported that sago effluent has potential to be a feedstock for aquaculture production and can be used as supplement for prawn feed (Awg-Adeni *et al.*, 2010).

Several studies on the utilisation of sago hampas have been described in relation to fibrous and starch components in sago hampas. Awg-Adeni *et al.* (2012) stated that sago hampas has the potential to be used as as substrate for local microbes to produce enzyme and reducing sugars, manufacture of particleboard, for hydrolysis to confectioners' syrup, compost for mushroom culture and animal feed. Furthermore, the reducing sugars produced can readily be used as feedstock in the production of ethanol, fuel alcohol and other value chemicals (Bujang, 2014; Subashini *et al.*, 2011). As a conclusion, sago effluent and its constituents are potentially used as promising renewable and cheap feedstock for the production of bioethanol production as well as providing solution for management of waste (Kannan *et al.*, 2013).

2.3 Starch

Starch is the reserve carbohydrate used as a way to store excess glucose in different botanical origins. It represents the second most abundant biopolymer on earth, next to cellulose (Alc ázar-Alay & Meireles, 2015; Kim *et al.*, 2004; Schirmer *et al.*, 2013). This renewable

natural resource is synthesised through photosynthesis by plants as energy and carbon sources. Starch production is performed diurnally and rapidly in the chloroplasts by plants. Conversely, starch that is synthesised in amyloplasts and organelles is responsible for the starch reserve synthesis of tubers, seeds and roots that are deposited for long term used (Alc ázar-Alay & Meireles, 2015). As early as the last century, starch plays an important role as functional material in the food (production of high-fructose corn syrups, glucose and glucose syrups) and non-food processing industries such as paper, chemical, pharmaceutical, textile and candy manufacture (Beninca *et al.*, 2008; van der Maarel *et al.*, 2002). Additionally, starch also serves as an essential source of energy for human and animal nutrition (Schirmer *et al.*, 2013).

Starch is a semi-crystalline polymer that is mainly composed of two homopolysaccharides which are amylose and amylopectin (Kim *et al.*, 2004; Provost *et al.*, 2016). According to Alc ázar-Alay & Meireles (2015), amylose consists 15%-30% whereas amylopectin constitutes 70%-85% common starch. Amylose is a straight glucose molecule chain polymer, which has sparse α -1,6-linked D-glucopyranose chains and mostly α -1,4-linked D-glucopyranose. The molecular weight of amylose is about 10^6 Da and forms a flexible molecule that can interact with alcohols, fatty acids and iodine (Cao *et al.*, 2015; Zou *et al.*, 2012). Amylopectin is also primarily a straight glucose molecule chain that joined by α -1,4 glycosidic bonds, but it is highly branched at every 24 to 30 glucose units consisting of α -1,6 bond (Alc ázar-Alay & Meireles, 2015; Ritslaid *et al.*, 2010). Its branched structure with high molecular weight, which is about 10^8 Da, interfere with any tendency to form significant levels of closely oriented hydrogen bonding and reduce the mobility of the polymer chains (Zou *et al.*, 2012). A matrix of starch granules with alternating amorphous of amylose and

crystalline of amylopectin material can be formed by arranging both polymers in a semi-crystalline structure (Alc ázar-Alay & Meireles, 2015; Zou *et al.*, 2012). The amylose-amylopectin ratio in starch granules as well as degree of branching in amylopectin define the phase transition behaviours and rheological properties (Aydemir *et al.*, Schirmer *et al.*, 2013). The structure and components of starch is shown below (Figure 2.4).

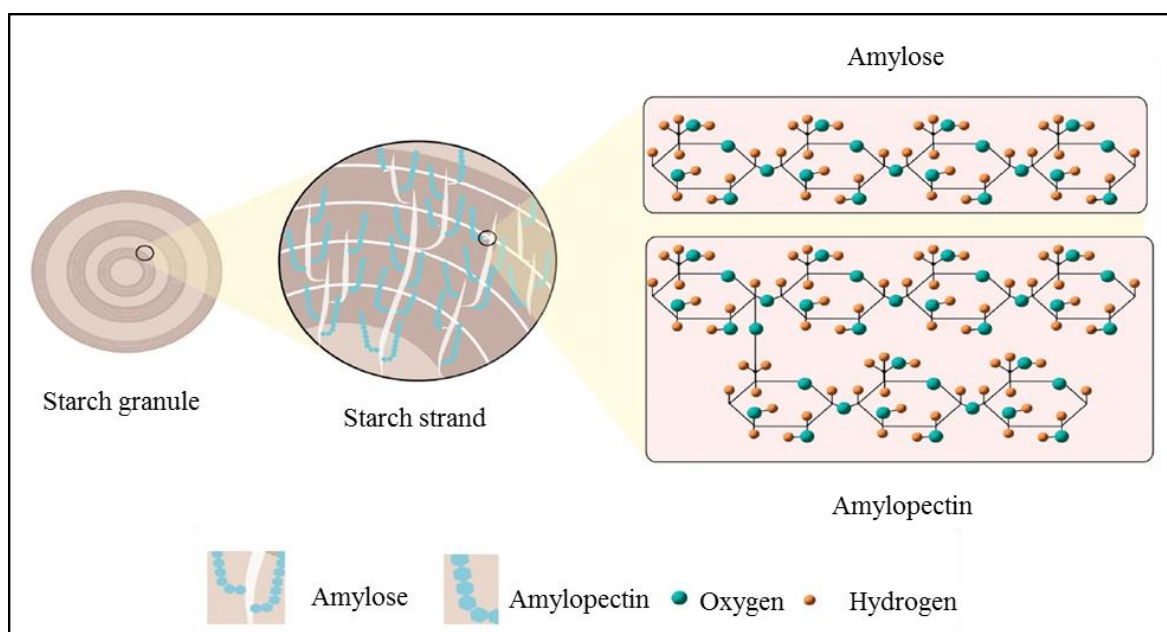


Figure 2.4: The structure and components of starch (Aydemir *et al.*, 2014).

Several changes at molecular and structural level occur when starch is heated in the presence of excess water, through transition phase termed as gelatinisation (Alc ázar-Alay & Meireles, 2015; Schirmer *et al.*, 2013). Gelatinisation stage occurs when water begins to dissolve starch by interacting via hydrogen bonds with the -OH groups of starch molecules at 60 to 70 °C, which then causes starch granules become gel-like solution thick with swelled starches. It is outcome of the amorphous phase hydration causing loss of crystallinity and molecular order (Alc ázar-Alay & Meireles, 2015; Provost *et al.*, 2016; Ritslaid *et al.*, 2010). Gelatinised starch can be easily hydrolysed to yield individual fermentable sugars such as

glucose and maltose molecules by enzymatic activities. After that, the resulting fermentable sugars can be utilised to produce ethanol with the aid of yeast (Kim *et al.*, 2004; Ritslaid *et al.*, 2010). The starch hydrolysis and ethanol fermentation take place according to the scheme shown in Figure 2.5.

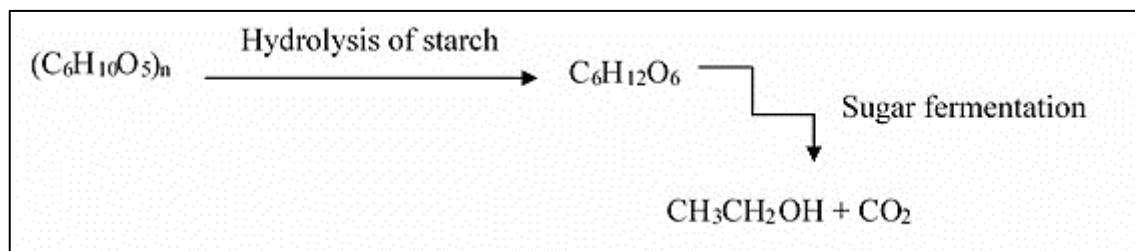


Figure 2.5: Conversion of starch into ethanol (Ritslaid *et al.*, 2010).

2.4 Lignocellulosic Biomass

Lignocellulosic biomass refers to the complex biological material that is cheap and is available from plants (Naik *et al.*, 2010). These may be categorised based on sources such as crop residues (sugarcane bagasse, oat straw, wheat straw, rice straw, corn stover, rye straw and pulps), forestry waste (grass, hardwood, softwood), cellulose wastes (newsprint, waste paper, recycled paper) and municipal solid wastes (MSW) (Behera *et al.*, 2014; Verma *et al.*, 2011). Figure 2.6 shows some common lignocellulosic biomasses.



Figure 2.6: Lignocellulosic biomasses (Anwar *et al.*, 2014).

Lignocellulosic biomass is common discarded for burning, which it is not only restricted to developing countries (Anwar *et al.*, 2014). Recently these inedible feedstocks are recognised as vast renewable energy resources and gained increased research interests. These abundant biomasses can be further exploited into high value products including value added fine chemicals, biofuel particularly bioethanol and cheap energy sources for microbial fermentation and enzyme production (Anwar *et al.*, 2014; Li *et al.*, 2016). Consequently, various approaches have been proposed for processing of lignocellulosic biomass into efficient and economically viable value-added biotechnological products as shown in Figure 2.7.

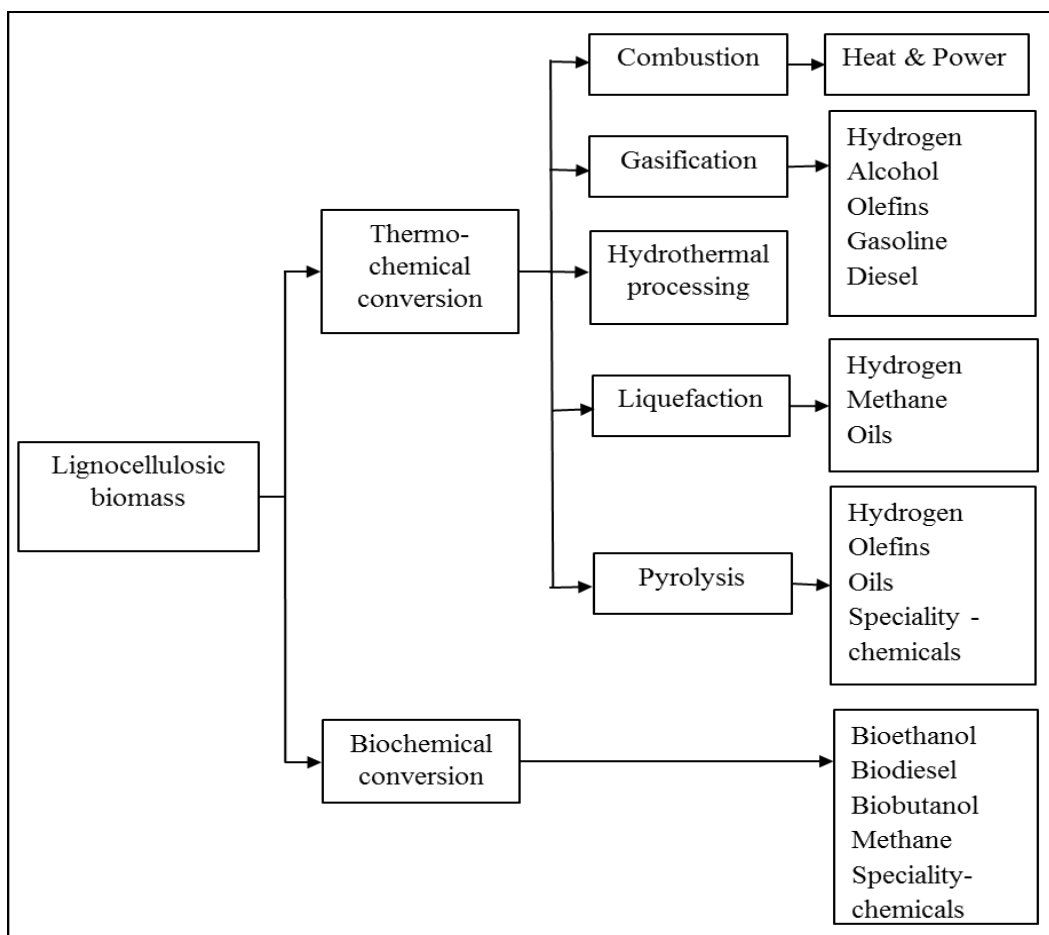


Figure 2.7: The conversion of lignocellulosic biomass into various values added biotechnological products (Anwar *et al.*, 2014).

Lignocellulose materials consist mainly of structural carbohydrates such as cellulose and hemicelluloses, and heterogeneous phenolic polymer lignin as its primary components (Zhao *et al.*, 2012; Verma *et al.*, 2011). Apart from that, some lignocellulosic biomass also consists of starch or known as starchy-lignocellulosic biomass (Awg-Adeni *et al.*, 2010; Diong *et al.*, 2016). Typically, most lignocellulosic materials are comprised of 40.0%-60.0% cellulose, 20.0%-40.0% hemicellulose and 10.0%-30.0% lignin (Anwar *et al.*, 2014; Kang *et al.*, 2014; Verma *et al.*, 2011). However, the contents of lignocellulosic biomass vary substantially according to the species, variety, climate, soil fertility and fertilisation practices (Zhao *et al.*, 2012). The compositions of cellulose, hemicellulose, lignin and ash in various

lignocellulosic biomasses are shown in Table 2.2. Figure 2.8 and Figure 2.9 show the schematic diagram of plant cell walls and its major components.

Table 2.2: The composition of cellulose, hemicellulose and lignin in lignocellulosic biomasses.

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Perennial grass	37.0-45.0	17.0-21.0	19.0-25.0	Sorek <i>et al.</i> (2014)
Pine (softwood tree)	25.0-42.0	18.0-26.0	21.0-30.0	Sorek <i>et al.</i> (2014)
Poplar (hardwood tree)	40.0-55.0	18.0-25.0	24.0-40.0	Limayen & Ricke (2012); Sorek <i>et al.</i> (2014)
Arid climate succulent	31.0-55.0	7.0-12.0	8.0-17.0	Sorek <i>et al.</i> (2014)
Nut shells	25.0-30.0	25.0-30.0	30.0-40.0	Anwar <i>et al.</i> (2014)
Rice hulls	24.0-36.0	12.0-19.0	11-19	Paulov á <i>et al.</i> (2013)
Rice straw	36.2	19.0	9.9	Mussatto & Teixeira (2007); Paulov á <i>et al.</i> (2013)
Corn cobs	45.0	35.0	15.0	Lee <i>et al.</i> (2014); Anwar <i>et al.</i> (2014)
Corn stover	38.0	26.0	19.0	Anwar <i>et al.</i> (2014); Singh <i>et al.</i> (2014)
Corn straw	42.6	21.3	8.2	Paulov á <i>et al.</i> (2013)
Cotton stalks	58.5	14.4	21.5	Mussatto & Teixeira (2007)
Soya bean	33.0	14.0	-	Singh <i>et al.</i> (2014)
Oat straw	39.4	27.1	17.5	Mussatto & Teixeira (2007)
Wheat straw	38.0	29.0	15.0	Singh <i>et al.</i> (2014)
Rye straw	31.0	25.0	-	Singh <i>et al.</i> (2014)
Barley straw	42.0	28.0	-	Singh <i>et al.</i> (2014)
Sugarcane bagasse	40.0	27.0	10.0	Mussatto & Teixeira (2007); Paulov á <i>et al.</i> (2013)
Sunflower stalks	42.1	29.7	13.4	Mussatto & Teixeira (2007)
Empty fruit brunch	41.0	24.0	21.2	Lee <i>et al.</i> (2014)
Switch grass	37.0	29.0	19.0	Paulov á <i>et al.</i> (2013); Singh <i>et al.</i> (2014)
Indian grass	39.0	29.0	-	Singh <i>et al.</i> (2014)
Miscanthus	43.0	24.0	19.0	Singh <i>et al.</i> (2014)
Reed canary grass	24.0	36.0	0	Singh <i>et al.</i> (2014)
Sweet sorghum	23.0	14.0	11.0	Singh <i>et al.</i> (2014)
Pearl millet	25.0	35.0	0	Singh <i>et al.</i> (2014)
Sago hampas	23.0	9.2	4.0	Awg-Adeni <i>et al.</i> (2012); Thangavelu <i>et al.</i> (2014)
Chemical pulps	60.0-80.0	20.0-30.0	2.0-10.0	Paulov á <i>et al.</i> (2013)
Newspaper	40.0-55.0	25.0-40.0	18-30	Anwar <i>et al.</i> (2014); Kang <i>et al.</i> (2014)
Paper sludge	33.0-61.0	14.2	8.4-15.4	Paulov á <i>et al.</i> (2013)
Magazine	34.3	27.1	14.2	Paulov á <i>et al.</i> (2013)
Cardboard	49.6	15.9	14.9	Paulov á <i>et al.</i> (2013)

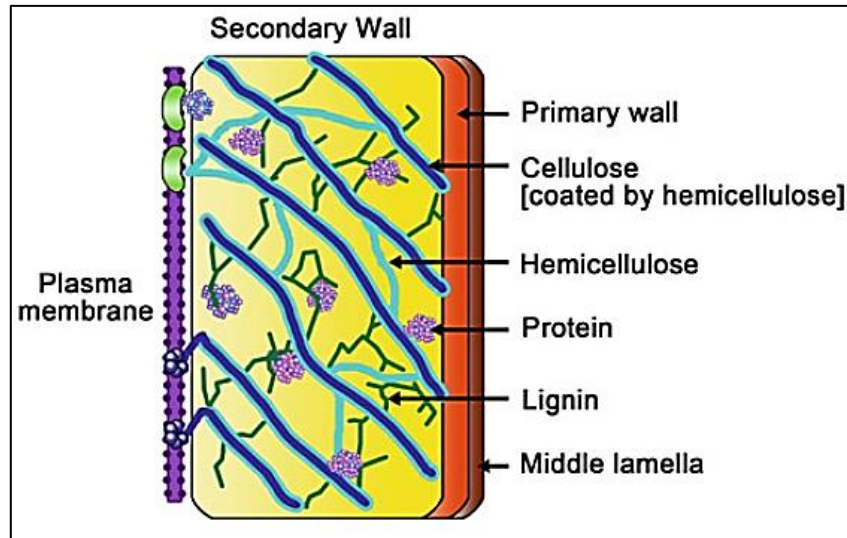


Figure 2.8: The schematic drawing of plant cell wall (Zhao *et al.*, 2012).

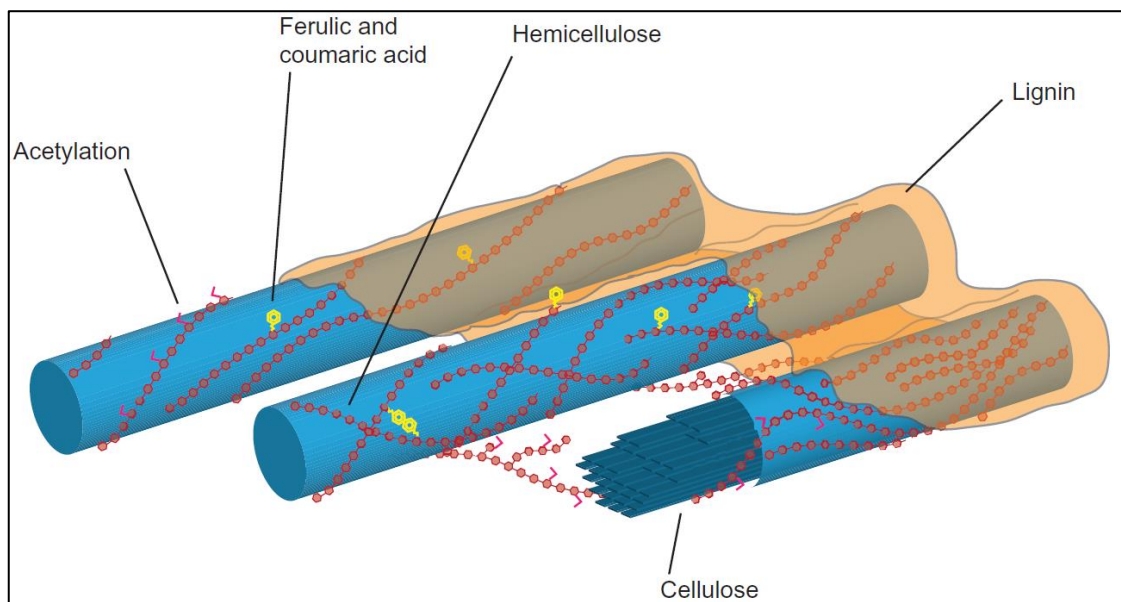


Figure 2.9: The major components in plant cell wall (Sorek *et al.*, 2014).

Lignocellulosic biomass has high potential for ethanol production compared to first-generation bioethanol, because it is a widely available cheap feedstock and is not in the human food chain (Ritslaid *et al.*, 2010). Limayen and Ricke (2012) stated that there are four stages included in the production of lignocellulosic-based ethanol, which are pretreatment, hydrolysis, fermentation and distillation. The pretreatment process is known to be the most

difficult in the conversion of lignocellulosic biomass, to break down structural and chemical biomass complex (Ritslaid *et al.*, 2010). Cellulose and hemicellulose in biomass are then hydrolysed into fermentable sugars (Figure 2.10), followed by further recovery of ethanol by well-known and elaborated fermentation technologies (Aydemir *et al.*, 2014; Kang *et al.*, 2014; Ritslaid *et al.*, 2010).

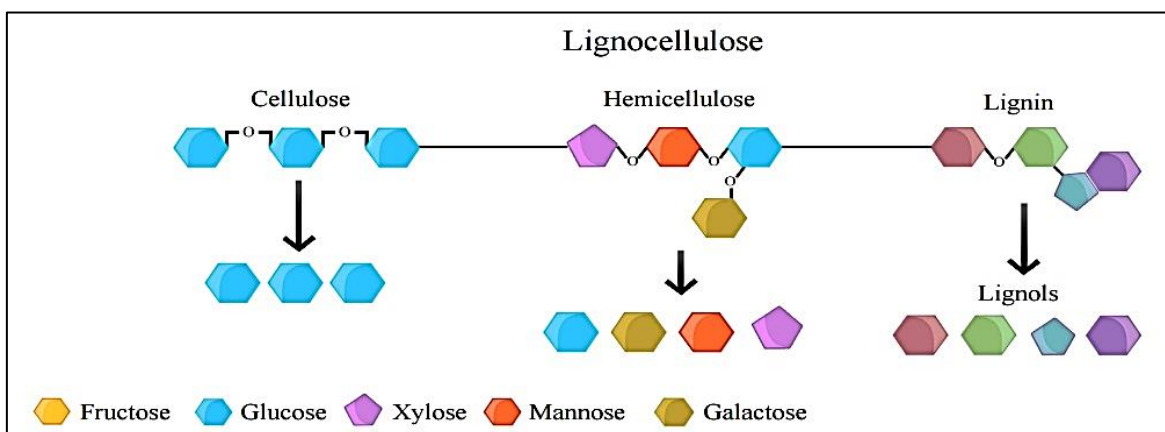


Figure 2.10: Hydrolysis of lignocellulose into fermentable sugars (Aydemir *et al.*, 2014).

2.5 Cellulose

Cellulose is found in a wide variety of living species such as plants, animals, and some bacteria. It is the most abundant biopolymer on Earth (Khalil *et al.*, 2014; Lavoine *et al.*, 2012). It is a major structural component of plant cell wall which provides mechanical strength and chemical stability to plants (Limayen & Ricke, 2012; Singh *et al.*, 2014; Sorek *et al.*, 2014). da Costa Lopes *et al.* (2013) described that cellulose chains are embedded in the core of the lignocellulosic biomass with hydroxyl groups oriented to form strong intra- and intermolecular hydrogen bonds. The chemical formula of cellulose is $(C_6H_{10}O_5)_n$ and its chemical structure is shown in Figure 2.11.

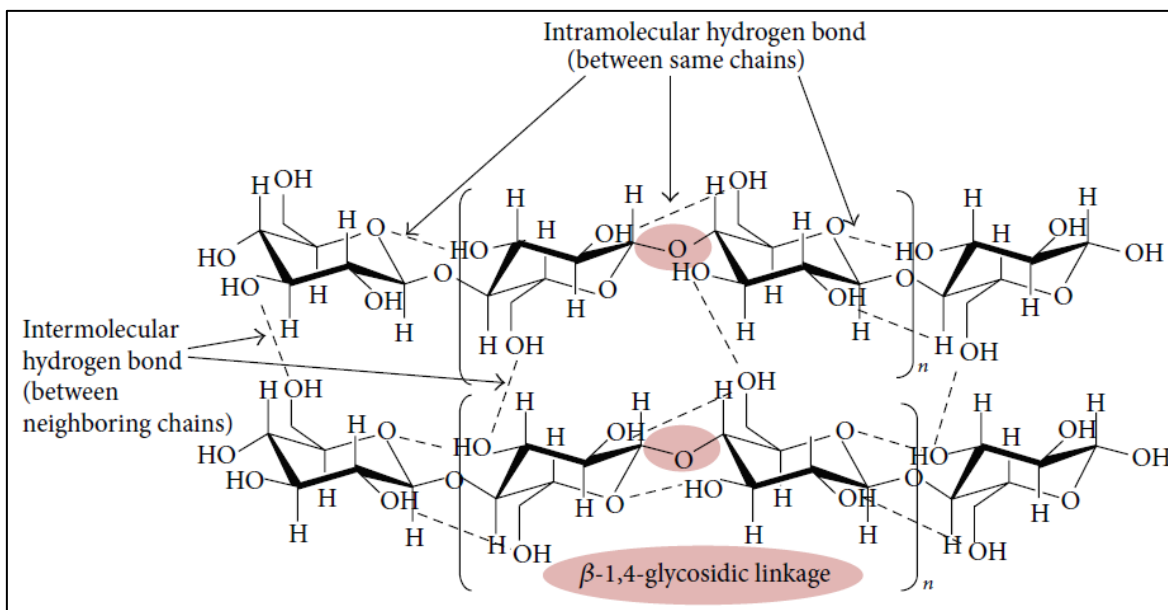


Figure 2.11: Chemical structure of cellulose chain (Lee *et al.*, 2014).

Cellulose is a linear polymer consisting of D-glucose subunits linked together by β -(1,4)-glycosidic bonds with a degree of polymerization (DP) and can vary from 500 to 15,000 glucose molecules, depending on the type of tissue (Elbinger *et al.*, 2016; Kumar *et al.*, 2009; Sorek *et al.*, 2014). According to Lavoine *et al.* (2012), DP for cellulose chains is approximately 10,000 whereas for native cellulose cotton, the DP is 15,000. Cellobiose, which consists of two molecules of glucose, is the repeating disaccharide unit built through this linkage. Cellulose chains are parallel where neighbouring glucose molecules in the chain are rotated 180° in relation to the adjacent molecules (Harmsen *et al.*, 2010).

There are extensive hydrogen bonds and van der Waals forces among cellulose molecules that lead to a crystalline and strong matrix. These bonds cause the cellulose to be packed into microfibrils as shown in Figure 2.12 (Kang *et al.*, 2014; Sorek *et al.*, 2014). According to Limayen and Ricke (2012), the presence of the microfibrils make the molecule stronger and

compactness. Hence, cellulose microfibrils has both highly compact crystalline region and less-ordered amorphous region (Mussatto & Teixeira, 2007).

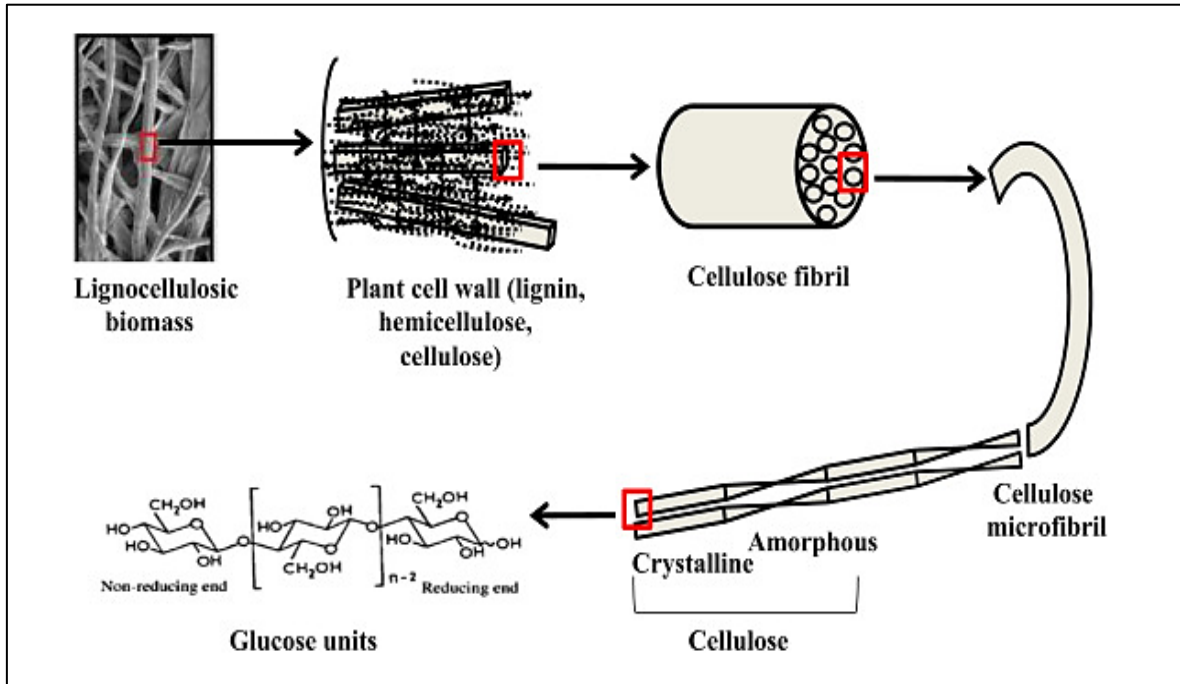


Figure 2.12: Structure of cellulose microfibrils (Kuhad *et al.*, 2016).

Cellulose originating from different plants differ in crystalline structure and inter-connections between other biomass components (Paulová *et al.*, 2013). The strong inter-chain hydrogen bonds among the hydroxyl groups of glucose residues in radial orientation and the aliphatic hydrogen atoms in axial positions create a semi-crystalline structure resistant to enzymatic hydrolysis. Additionally, due to the weaker hydrophobic interactions between cellulose, the water layer formed near the cellulose surface can protect the depolymerisation of cellulose via acid hydrolysis (Paulová *et al.*, 2013). Hence, the orientation of the linkage and addition of hydrogen bonding make the polymer rigid and difficult to break (Hamelinck *et al.*, 2005).

First-generation biofuel has been under close scrutiny due to the several limitations and thus, leading to the development of next generation biofuel production (Sims *et al.*, 2010). Thus, the abundance of cellulosic biomasses are introduced to be hydrolysed into fermentable sugars by using the combination actions of endo- and exoglucanase, glucosidases, and polysaccharide mono-oxygenases, as a prerequisite for the subsequent production of cellulosic ethanol (Hamelinck *et al.*, 2005; Kumar *et al.*, 2008; Sorek *et al.*, 2014).

2.6 Hemicellulose

Hemicellulose ($C_5H_8O_4$)_n, is the second most abundant heteropolysaccharide. It is a general designation for cell wall polysaccharides that are neither cellulose nor pectin (Anwar *et al.*, 2014; Badiei *et al.*, 2014; Sorek *et al.*, 2014). Hemicellulose functions as load bearing and cross-linking agent in the wall, binding structural (cellulose bundles, lignins, cell wall proteins, pectins) and nonstructural polysaccharides through a variety of covalent and noncovalent interaction (Sorek *et al.*, 2014). Hence, secondary cell wall hemicellulose provides structural backbone to plant cell wall (Badiei *et al.*, 2014).

This branched structure consists of pentoses (D-xylose, L-arabinose) as well as hexoses (D-galactose, D-glucose, D-mannose). Besides that, it may also contain acetylated sugars such as D-glucuronic, D-galacturonic and methylgalacturonic acids derived from pectin or pectic acids (da Costa Lopes *et al.*, 2013; Limayem & Ricke, 2012; Paulova *et al.*, 2013). Limayem and Ricke (2012) reported that the backbone chain of hemicellulose is primarily composed of xylan β (1,4)-linkages that include nearly 90.0% D-xylose and approximately 10.0% L-

arabinose. However, the frequency and composition of branches vary greatly on the type of materials from the aspects of the nature and source of feedstock (Limayem, & Ricke, 2012; Paulova *et al.*, 2013). According to Limayen and Ricke (2012), hemicelluloses in hardwood are typically xylans (Figure 2.13A), while the softwood hemicelluloses are more frequently composed of glucomannans (Figure 2.13B). Unlike cellulose, amorphous hemicelluloses are easily converted into monomer sugars via hydrolysis (Zhao *et al.*, 2012).

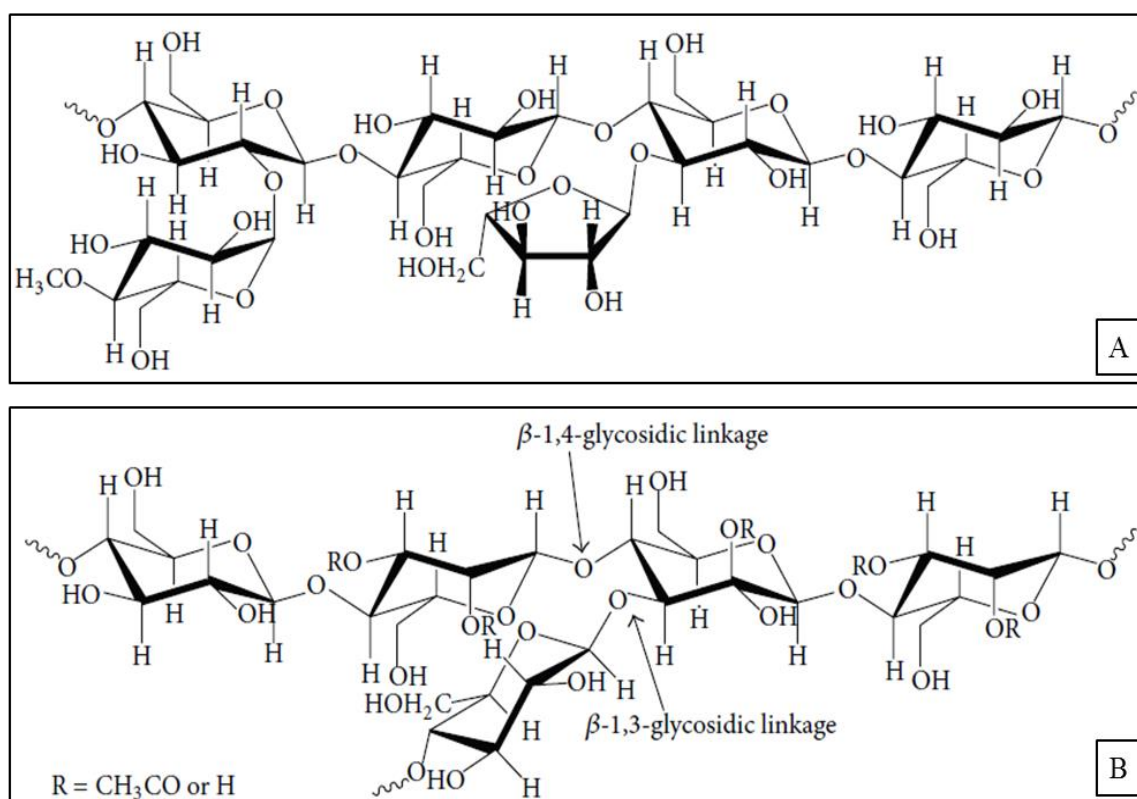


Figure 2.13: Chemical structure of hemicellulose compounds (A) xylan and (B) glucomannan (Lee *et al.*, 2014).

In typical lignocellulosic biomass, cellulose fibrils and lignin are interlaced and embedded within a matrix of hemicelluloses, which significantly increase the strength and toughness of plant cell walls (Zhao *et al.*, 2012). The major hemicelluloses in plant biomass are xyloglucan and xylan. Xyloglucan is located abundantly in the primary walls, while xylan,

also known as glucuronoxylans are the dominant non-cellulosic polysaccharides in the secondary walls of dicots. The schematic illustration of xyloglucan and xylan is shown in Figure 2.14. Hence, the major sugars that existed in the hydrolysate of hemicelluloses are xylose, glucose, galactose and arabinose (Zhao *et al.*, 2012).

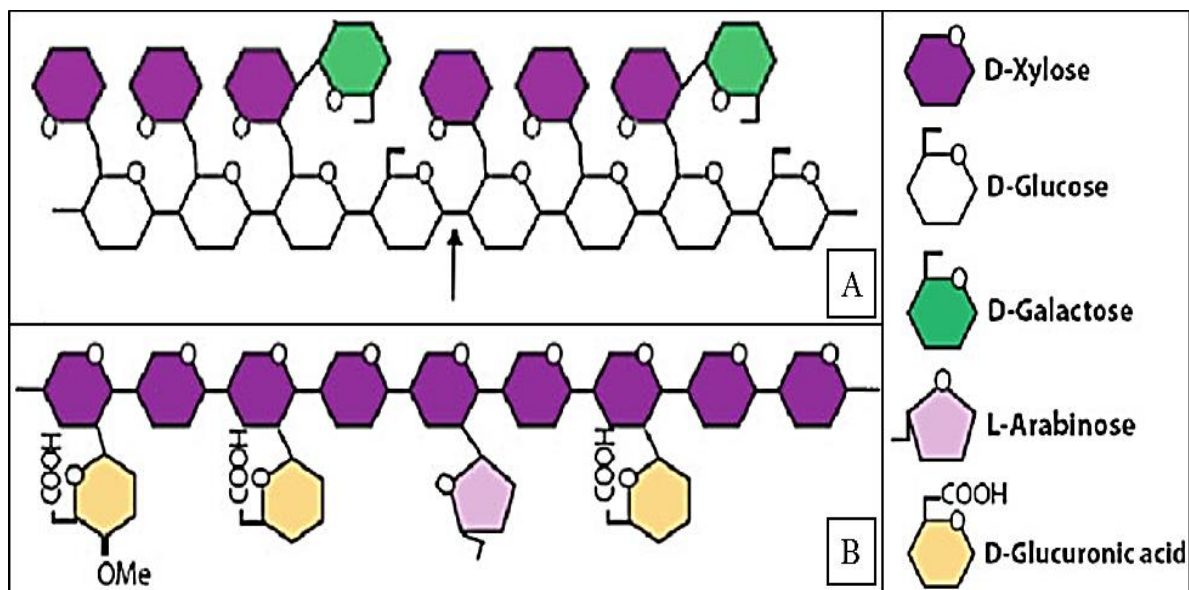


Figure 2.14: Schematic illustration of (A) xyloglucan, arrow indicates the typical cleavage site and (B) xylan, typical dicot structure (Zhao *et al.*, 2012).

Traditionally, hemicellulose serves as a major source of food and nutrients for herbivores and as feedstock for the production of food, textiles, paper, and pulp industries. Like cellulose, hemicellulose can be depolymerized into simple form of fermentable sugars by enzymatic hydrolysis with the action of hemicellulase. However, the conversion of pentose sugar into ethanol creates a challenge due to the unavailability of robust ethanol metabolized by most microbes, especially the common industrial yeast (Chandel *et al.*, 2010).

2.7 Lignin

Lignin is a heterogeneous polymer comprised of complex and large polymer of phenyl-propane, methoxy group and non-carbohydrate poly phenolic substance (Anwar *et al.*, 2014). The main block of the lignin is phenyl-propanes unit. Three phenyl propionic alcohols that exist as monomers of lignin are *p*-coumaryl, coniferyl and sinapyl alcohol. These are held together by different kinds of linkages, but most commonly linked by ether bonds (Anwar *et al.*, 2014; Hendriks & Zeeman, 2009). The chemical structure of lignin is shown in Figure 2.15.

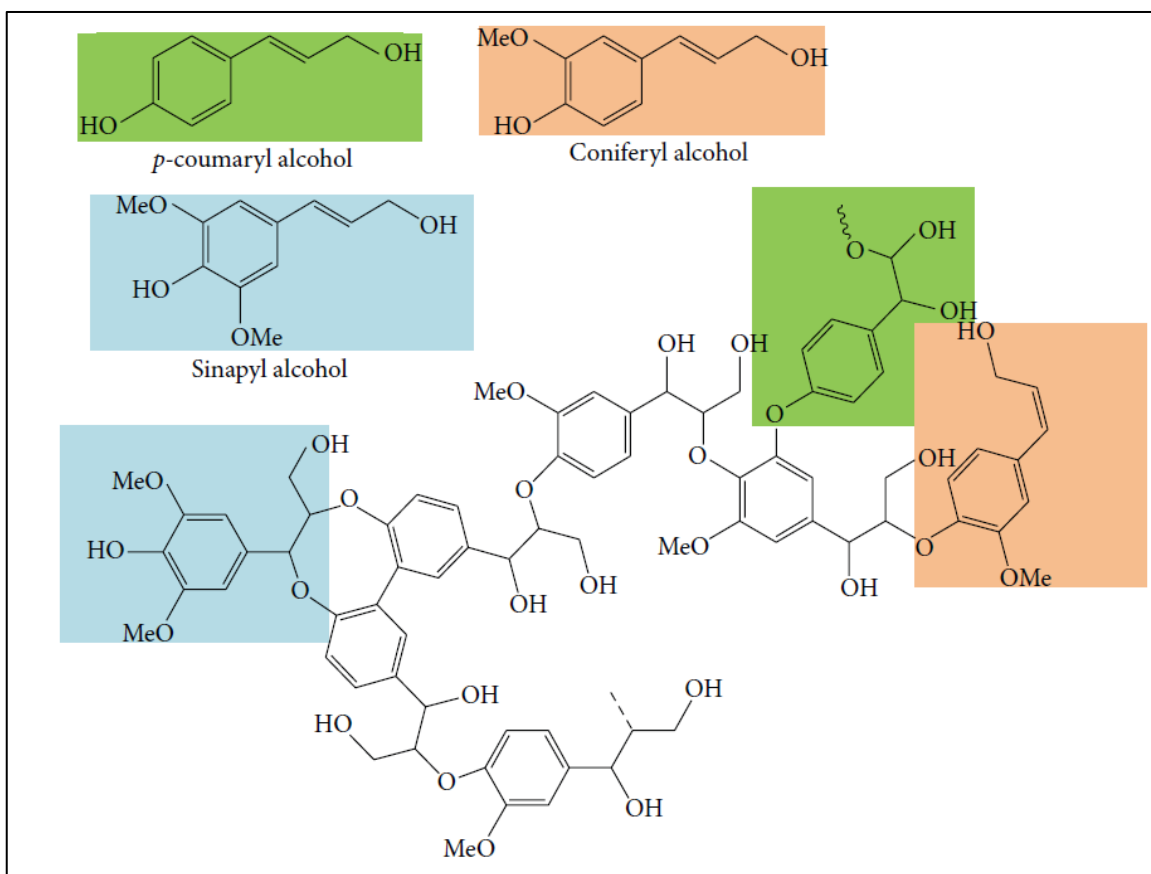


Figure 2.15: Chemical structure of lignin (Lee *et al.*, 2014).

Like cellulose and hemicellulose, lignin that is present in the cellular wall is one of the most abundant polymers in nature (Hendriks & Zeeman, 2009). It is insoluble in water, making the degradation of lignin very hard. The functions of lignin are to provide rigidity and cohesion to the plant cell wall, confer water impermeability to xylem vessels, and resistance against microbial attack and oxidative stress (Mussatto & Teixeira, 2007). In addition, this complex material acts like a glue by filling the gap between and around the cellulose and hemicellulose complex with the polymers (Anwar *et al.*, 2014). According to Wi *et al.* (2015), lignin is believed to be a major hindrance to enzymatic degradation on carbohydrates, as its close association with the microfibrils prevent the enzyme activities. Hence, a variety of lignin removal or delignification of biomass greatly increases digestibility to increase cell wall porosity, minimize cellulose degradation, and thus, render the biomass more amenable to enzymatic hydrolysis (Raud *et al.*, 2015; Wi *et al.*, 2015).

Lignocellulosic biomass with a high lignin content is not readily applicable as raw material for the ethanol production (Kang *et al.*, 2014). Currently, various chemical, physical and biological pretreatment have been proposed to remove lignin from plant biomass, but only a few are promising, such as steam explosion, ammonia fiber expansion (AFEX), dilute acid, and ionic-liquid pretreatment (Anwar *et al.*, 2014; Saini *et al.*, 2015; Wi *et al.*, 2015). The removal of lignin is necessary as the phenolic groups formed from the lignin degradation can substantially deactivate cellulolytic enzymes and hence, influence enzymatic hydrolysis (Kang *et al.*, 2014). Furthermore, the various enzyme inhibitors or deactivators and toxic substances produced during pretreatment, may also inhibit the later fermentation processes, leading to lower ethanol yields and productivities (Raud *et al.*, 2015). Hence, the

pretreatment stage is deemed as the main processing challenge and efforts are currently focused in optimising several of these procedures (Saini *et al.*, 2015; Srivastava *et al.*, 2015).

2.8 Biorenewable Energy

The availability, supply and use of energy play a central role in the way societies organise themselves - from individual welfare to social and industrial development. Moreover, energy accessibility and costs are determining factors for the economic, political and social interrelations among nations. As stated by Sarkar *et al.* (2012), the worldwide economic is highly dependent on various fossil energy sources. Fossil fuel has been used for the production of fuel, electricity and other benefits. However, the continuous use of fossil fuels to fulfil the energy demand had led to the serious environmental concerns (Sarkar *et al.*, 2012). In response to serious worldwide economic and environmental pollution issues caused by the excessive use of fossil fuel, considerable attentions are now focused on the research and development of biorenewable energy, particularly biofuel (Chin & H'ng, 2013; Thangavelu *et al.*, 2014).

Biofuel is defined as any types of fuel (solid biomass, liquid fuels and biogases) whose energy is predominantly derived from natural materials (Chin & H'ng, 2013; Shah & Sen, 2011). Ethanol, methanol, biodiesel, and Fisher-Tropsch diesel are classified as liquid biofuel whereas hydrogen and methane are gaseous biofuels (Demirbas, 2008). Liquid and gaseous biofuel can be produced via two main ways, which are thermochemical and biological in nature (Demirbas, 2008). Biofuels offer many technical and environmental

benefits over conventional fossil fuels. The major benefits of this biorenewable energy are noted in Table 2.3. However, the most obvious benefit of biofuel is that much of the carbon dioxide released during combustion of biofuel is taken in photosynthesis process (Fink & Medved, 2011; Shah & Sen, 2011). Oxygen state is stated as the biggest difference between biofuels and petroleum feedstocks. The oxygen level of biofuel is from 10.0% to 45.0%, while petroleum has essentially none (Demirbas, 2008). All of these advantages make biofuels more economically feasible, by reducing dependence on petroleum and non-renewable energy sources (Izmirlioglu & Demirci, 2012).

Table 2.3: Major benefits of biofuels (Demirbas, 2009).

Economic impacts	Sustainability
	Fuel diversity
	Expanded number of rural manufacturing jobs
	Expanded income taxes
	Enhanced investment
	Agricultural development
	Minimising the dependency on imported petroleum
Environmental impacts	Greenhouse gas reductions
	Air pollution reduction
	Biodegradability
	Higher combustion efficiency
	Enhanced land and water use
	Carbon sequestration
Energy security	Domestic distribution
	Supply reliability
	Minimizing use of fossil fuel
	Ready availability
	Renewability

Based on the basics of production technologies, biofuels can be categorised into biofuels of first, second, third and fourth generation. First-generation biofuels refer to biofuel made from plants rich in sugar or oil (Ritslaid *et al.*, 2010). There are three main types of first-generation biofuels used commercially, which are made from sugar- and starch-based ethanol, oil-crop

based biodiesel and straight vegetable oil, as well as biogas derived through anaerobic digestion (Chin & H'ng, 2013; Naik *et al.*, 2010). However, discarding of cellulose and hemicellulose in the production of first-generation biofuel is economically unreasonable as these are constituted most of the carbon resource of the plants (Ritslaid *et al.*, 2010). Additionally, the use of food crops such as corn, sugarcane and palm oil as feedstock in its production is very controversial as this practice has to compete with food products intended for human consumption. Consequently, this competition resulted in an increase in the price of food grains and other allied products (Searchinger *et al.*, 2015; Welker *et al.*, 2015). Hence, the production of first-generation biofuels is costly as well as limited by the concerns about environmental impacts and carbon balance (Karmee *et al.*, 2016; Naik *et al.*, 2010). Therefore, novel biofuels are introduced as second-generation biofuels (Ritslaid *et al.*, 2010; Vincent *et al.*, 2015b).

With advanced technology, second-generation biofuels are derived from lignocellulosic biomasses such as agricultural residues (cane, bagasse, wheat straw, corn stalk), forestry wastes (chips and sawdust from lumber mills, dead trees, tree branches), municipal solid waste (household garbage, paper industry), energy crops (switchgrass), food processing and other industrial wastes (black liquor, sago effluent) (Awg-Adeni *et al.*, 2010; Chin & H'ng, 2013; Shah & Sen, 2011). According to Naik *et al.* (2010), second-generation biofuels do not compete with food crops as they utilise mainly cheap and abundant materials available from plants. It is the most cost-effective route to renewable and low carbon energy as transportation fuel compared to standard petrol and diesel (Naik *et al.*, 2010). Third and fourth generation biofuels are derived from algae by using modern gene and

nanotechnologies (Ritslaid *et al.*, 2010). The overview of biofuels of the four generations, including respective feedstock and technological processes, are shown as per Table 2.4.

Table 2.4: Type of biofuels in four generations with biomass feedstock and production process (Ritslaid *et al.*, 2010).

First-generation biofuel			
Type of biofuel	Name	Biomass feedstock	Production process
Bioethanol	Conventional bioethanol	Sugar beet, sugar cane, sugar sorghum	Hydrolysis & fermentation
Pure plant oil	Pure plant oil (PPO)	Oil plants (e.g. rape)	Cold-pressing/extraction
Biodiesel fuel (plant energy)	Rape methyl-/ethyl ester RME/REE	Oil plants (e.g. rape/turnip rape seeds, sunflower seeds, soy beans, etc.)	Cold-pressing/extraction/transesterification
Biodiesel fuel (waste grease)	Fatty acids methyl/ethyl ester (FAME/FAEE)	Biodiesel cooking and deep-fry grease	Transesterification
Biogas	Upgraded biogas	Wet biomass	Anaerobic digestion
Bio-ETBE		Bioethanol	Chemical syntheses
Second-generation biofuel			
Type of biofuel	Name	Biomass feedstock	Production process
Bioethanol	Cellulose ethanol	Lignocelluloses	Upgraded hydrolysis & fermentation
Synthesis biofuels	Mixed higher alcohols	Lignocelluloses	Gasification + syntheses
Biodiesel (hybrid biodiesel from the first and second generation)	NExBTL	Plant oils and animal fats	Hydrogenation (Refining/enrichment)
Biogas	SNG (Synthetic Natural Gas)	Lignocelluloses	Gasification & syntheses
Bio-hydrogen		Lignocelluloses	Gasification & syntheses or biology process
Third-generation biofuel			
Type of biofuel	Name	Biomass feedstock	Production process
Biodiesel	Oilgae Algae diesel	Algae	Gene and nanotechnology & esterification
Fourth-generation biofuel			
Type of biofuel	Name	Biomass feedstock	Production process
Bio-gasoline	Synthetic oil	Vegetable oil	Hydrolytic conversion/deoxygenating
Bio jet fuel Biodiesel		(CENTIA™ oil from algae)	

Liquid biorenewable fuel has attracted great attention in different countries as it is non-polluting, locally available, accessible, sustainable and reliable fuel (Demirbas & Dincer, 2008; Vasudevan *et al.*, 2005). In addition to fuel vehicles, liquid biofuel can also fuel engines or cells for electricity generation (Demirbas, 2008). The two most important liquid biofuels are centred on bioethanol and biodiesel which are derived mainly from vegetable oils, seeds and lignocelluloses as shown in Figure 2.16 (Fink & Medved, 2011; Hassan & Kalam, 2013). However, bioethanol is considered as most commercialised technology and most used biofuel on the global market (Chin & H'ng, 2013).

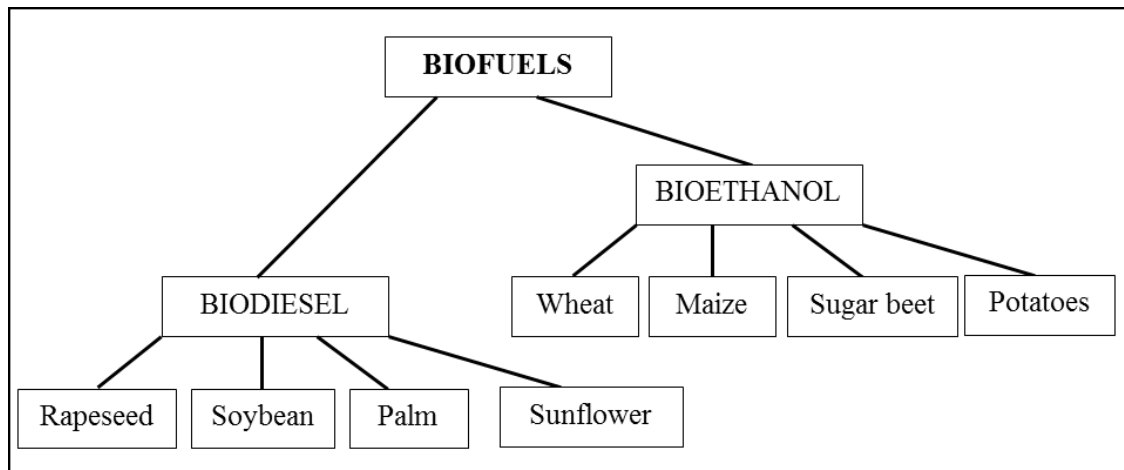


Figure 2.16: The resources of bioethanol and biodiesel (Demirbas, 2008).

2.9 Ethanol and Bioethanol

Ethanol is a volatile, flammable, clear and colourless liquid with an agreeable odour (Bajpai, 2013). It is commonly known as ethyl alcohol, grain alcohol, alcoholic spirit, grain spirit, absolute alcohol and ethyl hydrate (Ritsalaid *et al.*, 2010). In addition, it is biodegradable, low in toxicity, and causes little environmental pollution if spilt (Chin & H'ng, 2013;

Walker, 2010). Ethanol belongs to the alcohol group, a group of chemical compounds that contain a hydroxyl group, -OH (Bajpai, 2013). It is often abbreviated as EtOH, with a molecular structure shown in Figure 2.17. The term alcohol is derived from Arabic al-kuhul, which originally referred to any fine powder, but medieval alchemists later applied the term to refined product of distillation (Bajpai, 2013).

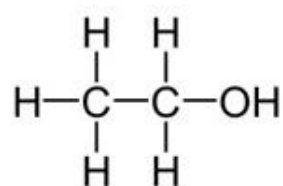


Figure 2.17: Chemical formula of anhydrous ethanol (Walker, 2010).

The physical properties of ethanol are dominantly attributed to the presence of both the hydroxyl group and the short carbon chain (Baeyens *et al.*, 2015). This functional group is prone to hydrogen bonding, enabling ethanol to be more viscous and less polar than any other organic compound with similar molecular weight. Ethanol is fully miscible in water as well as in most of organic solvents, making it useful as a solvent and as raw material for perfumes, paints, lacquer and explosives (Bajpai, 2013). The octane number of ethanol is high, which means the internal combustion engines involved ethanol can have a high compression ratio giving power output per cycle and increases resistance to engine knocking (Bajpai, 2013; Walker, 2010). Therefore, due to its physic-chemical properties, ethanol has a widespread usability in the alcohol industry as alcoholic beverages, tinctures and pharmaceutical preparations, solvents and preservatives, antiseptics and perfumes (Ritslaid

et al., 2010). The overall physic-chemical properties of anhydrous ethanol are shown in Table 2.5.

Table 2.5: The physic-chemical properties of anhydrous ethanol (Aditiya *et al.*, 2016; Li *et al.*, 2005; Ritslaid *et al.*, 2010; Walker, 2010).

Molecular Formula	C ₂ H ₅ OH or CH ₃ -CH ₂ OH
Molecular Weight	46.07 g/mol
Appearance	Colourless liquid (between -117.0 and 78.0 °C)
Water Solubility	Miscible
Density at 20 °C	0.789 × 10 ³ kg/m ³
Boiling Point	78.39 °C
Freezing Point	-114.15 °C
Pour Point	-117.3 °C
Flash Point (in closed cup)	13.0 °C
Ignition Temperature	425.0 °C
Refractive Index n at 20 °C	1.36048
Heat Content	26.80 MJ/kg
Explosion limits	Lower 3.5% v/v; upper 19.0% v/v
Vapour Pressure at 38 °C	50 mmHg
Higher Heating Value at 20 °C	29,800 kJ/kg
Lower Heating Value at 20 °C	21,090 kJ/L
Specific heat, Kcal/kg	60.0 °C
Acidity (pK _a)	15.9
Viscosity	1.19 mPa.s
Octane Number	99
Cetane Number	5-8
Carbon (wt)	52.1%
Hydrogen (wt)	13.1%
Oxygen (wt)	34.7%

Ethanol is produced via two different methods, which are 5% synthetically from petrochemical sources and 95% by microbial fermentation processes. Traditionally, ethanol is produced from glucose via anaerobic fermentation. Glucose is the simple sugar created in plants through photosynthesis process as shown by Equation 1 (Table 2.6). During the fermentation, glucose is converted into ethanol and carbon dioxide with the action of microorganisms (Equation 2 in Table 2.6). According to Ritslaid *et al.* (2010), the production

of ethanol by yeast is characterised by high selectivity, low accumulation of by-products, high ethanol yield, high fermentation rate, good tolerance toward both increased ethanol and substrate concentration and lower pH value. As depicted by Equation 3 (Table 2.6), ethanol burns completely to produce carbon dioxide, water and heat (Shah & Sen, 2011).

Table 2.6: Reactions in the production of ethanol from glucose (Shah & Sen, 2011).

Equation 1	$6 \text{ CO}_2 + 6\text{H}_2\text{O} + \text{light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6 \text{ O}_2$
Equation 2	$\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2 \text{ C}_2\text{H}_5\text{OH} + 2 \text{ CO}_2 + \text{heat}$
Equation 3	$\text{C}_2\text{H}_5\text{OH} + 3 \text{ O}_2 \rightarrow 2 \text{ CO}_2 + 3\text{H}_2\text{O} + \text{heat}$

Bioethanol is a domestically produced liquid fuel that has the same molecule as potable alcohol. It can be made by fermenting the sugar components of numerous renewable sources of feedstock, which are classified into three categories: sucrose-containing feedstocks, starch materials and lignocellulosic materials with conversion technologies (Balat, 2011; Ibeto *et al.*, 2011; Vohra *et al.*, 2014). Sucrose-containing feedstocks for bioethanol essentially comprise of sugarcane, sugar beet and sweet sorghum (Balat *et al.*, 2008). One of the world's largest ethanol producers, Brazil started mass production of bioethanol from sugarcane for more than 30 years (Chin & H'ng, 2013; Goldemberg, 2007). In the last decade, the use of sugarcane as a bioethanol crop in yielding anhydrous bioethanol and hydrated bioethanol has expanded much by fermentation and distillation of sugarcane juice and molasses. Furthermore, the conversion of sucrose into ethanol is easier compared to other feedstock due to the ability of disaccharide to be broken down into fermentable sugars without the action of enzyme (Cardona & Sanchez, 2007).

In North American and Europe, starchy materials such as corn, wheat and barley are the most utilised feedstock in the production of ethanol. Starch polymers are broken down in order to obtain glucose, which is converted into ethanol by yeast (Vohra *et al.*, 2014). Additionally, other starchy crops such as tubers and cassava can be used for the same purposes as well (Cardona & Sanchez, 2007; Vohra *et al.*, 2014). Through an enzymatic hydrolysis with amylolytic enzymes, glucose polymers in starch are broken into glucose and followed by fermentation, distillation and dehydration to yield anhydrous ethanol (Kumar *et al.*, 2011). There are two distinct methods for processing corn, which are wet milling and dry milling, and both operations can convert corn into ethanol (Lee & Shah, 2012).

With advanced biotechnology, lignocellulosic biomass such as agricultural residues, forestry wastes, municipal solid waste, energy crops, food processes and other industrial wastes were introduced as second-generation bioethanol feedstock (Cheng & Timilsina, 2011; Vohra *et al.*, 2014). Lignocellulosic biomass is made up of very complex sugar polymers and not generally used as food sources. Hence, it has advantage over other feedstocks, as it does not conflict with land-use for food production, and is cheaper than conventional bioethanol sources (Chin & H'ng, 2013). However, this biomass need to be converted into sugars before being fermented into ethanol (Shah & Sen, 2011).

As early as 1925, Henry Ford had promoted ethanol as “the fuel of the future” (Chandel *et al.*, 2007). Nowadays, bioethanol is considered as the most suitable petrol substitute for vehicles, justified by factors such as finite nature of fossil fuel, continuous increase of oil price and environmental pollution (Ibeto *et al.*, 2011). Unlike gasoline, the usage of bioethanol can reduce greenhouse gas emissions from fossil fuel with a zero theoretical net

contribution (Kim & Dale, 2005). As depicted in Figure 2.18, renewable resources like agricultural products and wastes, which obtain energy from sun, can be used as automotive fuels or motor fuel additive with different ratios in an existing transportation technology. As a result of the usage of ethanol in transportation engine, much of the carbon dioxide produced during the combustion of bioethanol is taken up by growing plants. Apart from that, the heat released from the combustion can be used to drive piston in the engine by expanding heated gases (Shah & Sen, 2011). Hence, Haagensen *et al.* (2005) documented that ethanol is an essentially unlimited renewable source of energy with a great potential of becoming the ‘green’ liquid fuel of the future.

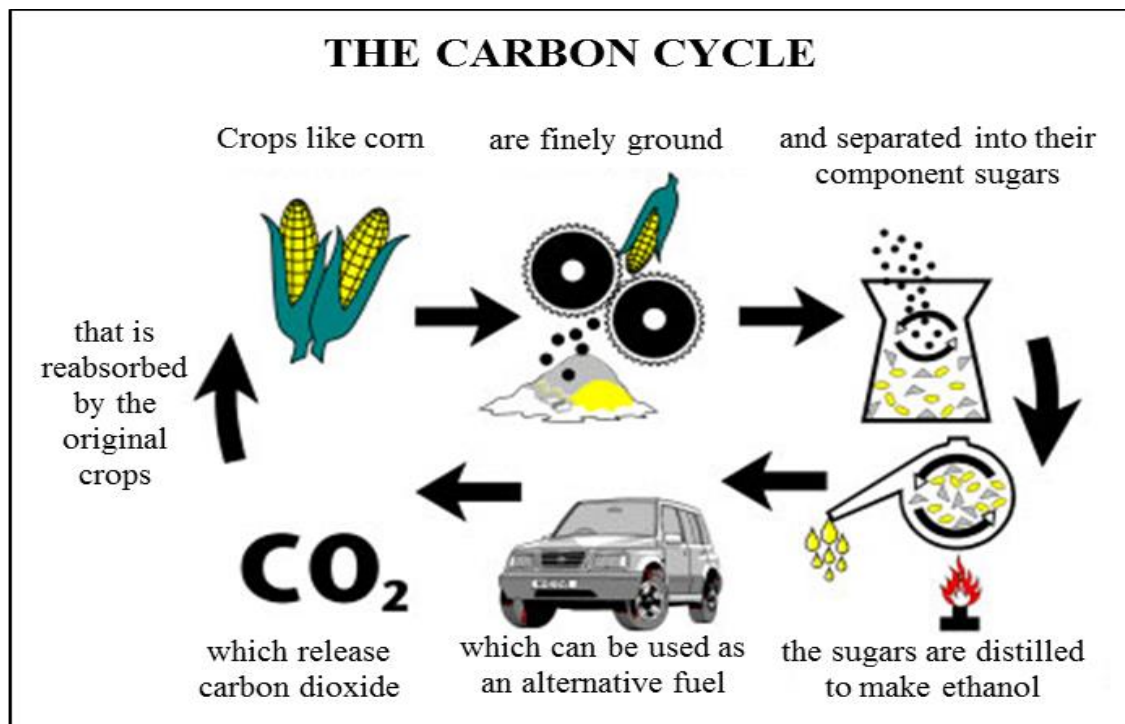


Figure 2.18: The carbon cycle in production of bioethanol (Shah & Sen, 2011).

2.10 Fuel Ethanol Production and Malaysian Perspective

The global fuel crisis of the early 1970s coupled with concerns for the environment have triggered the demand of biofuel, particularly bioethanol in many regions throughout the world (Limayem & Ricke, 2012; Ullah *et al.*, 2014). In response, several countries including United States, Brazil, Europe, China and Canada, were driven to develop their internal markets for biofuel as well as established plans for use of these biofuels (Mussatto *et al.*, 2010). Based on Figure 2.19, statistics of global ethanol industry has grown tremendously in response to surging domestic use and worldwide demand, from a little over 13,123 million gallons in 2007 to approximately 25,682 million gallons in 2015 (RFA, 2008; RFA, 2016). In 2016, this figure stood at 26,014 million gallons as shown in Table 2.5 (RFA, 2017). The two leading producers, United States and Brazil, accounted for about 86.0% of the total in 2015 (Table 2.7).

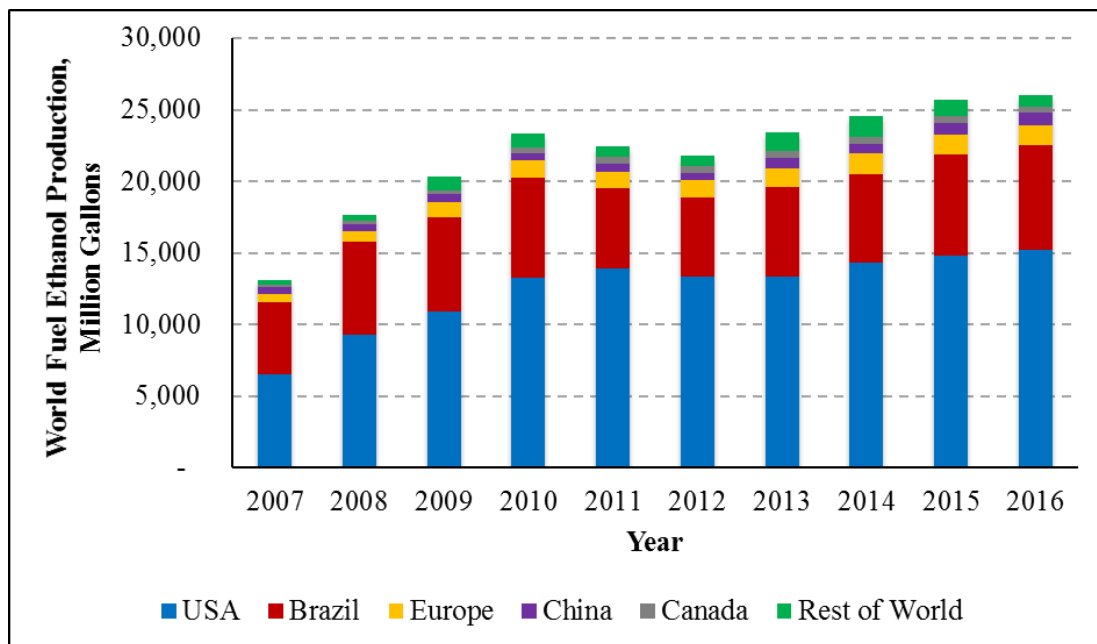


Figure 2.19: Global ethanol production by countries from 2007 until 2016.

Table 2.7: Global ethanol production in the year of 2016 (RFA, 2017).

Country	Millions of Gallons
USA	15,250
Brazil	7,295
Europe	1,377
China	845
Canada	436
Thailand	322
Argentina	264
India	225
World	26,014

Brazil was the first large-scale bioethanol producing country. It is also the front-runner in the use of renewable fuels in the world, with the inception of the National Program of Alcohol or PróAlcool (Chin & H'ng, 2013; Soccol *et al.*, 2010; Stambuk *et al.*, 2008). In 1975, the programme was implemented to substitute gasoline by ethanol as a response to the oil shock of 1973 and the decline in world sugar prices. Furthermore, methyl tertiary butyl ether (MTBE) used in gasoline was identified as an environment pollutant agent (Scarlat & Dallemand, 2011; Timilsina & Shrestha, 2011). Sugarcane was chosen as feedstock for ethanol production in this program, and as consequence, the agricultural and technological studies were greatly intensified as well as the substantial industry of sugar ethanol was developed (Soccol *et al.*, 2010; Tyner, 2008; Winchester & Reilly, 2015). Additionally, pure gasoline is no longer sold in Brazil as it is nationally blended with 20.0%-25.0% ethanol (Scarlat & Dallemand, 2011).

The United States of America is the largest consumer of petroleum products in the world by lending on imports to fulfil the demand (Dwivedi *et al.*, 2009). In 2016, the US consumed an average of 19.69 million barrels per day (7.21 billion barrels per year) of petroleum products, out of which about 51.3% or 10.10 million barrels per day was imported (Energy

Information Administration, 2017a; Energy Information Administration, 2017b). It is predicted that the consumption of gasoline as primary energy source for meeting non-commercial transportation demand will further rise along with the increasing population (Dwivedi *et al.*, 2009). Hence, the US stirred into action its corn-based ethanol program in which the role of renewable energy sources is emphasized (Timilsina & Shrestha, 2011). Currently, the US ethanol industry is the largest in the worldwide with Brazil a fading second. In 2016, the US led ethanol production with 15.25 billion gallons and Brazil at about 7.29 billion gallons (RFA, 2017). According to Urbanchuk (2016), the ethanol industry in the United States comprised approximately of 214 plants in 29 states with the total production capacity of 15.5 billion gallons as more than 160 million gallons of capacity were under expansion or construction at the end of 2015. The top ten ethanol producer by states in the United States is shown in Table 2.8.

Table 2.8: Top ten ethanol production capacity by states in the United States (RFA, 2017).

States	Production capacity	Operating	Under construction/ expansion	Millions of gallons
Iowa	4,072	4,016	-	4,072
Nebraska	2,182	2,129	10	2,192
Illinois	1,785	1,747	-	1,785
Minnesota	1,204	1,164	-	1,204
Indiana	1,173	1,173	-	1,173
South Dakota	1,059	1,059	-	1,059
Kansas	552	502	-	5,252
Wisconsin	547	547	-	547
Ohio	548	548	-	548
North Dakota	475	475	-	475

Apart from Brazil and United States, significant production increases are recorded in China (Figure 2.19). Canada, Thailand, Argentina, India, Australia, Thailand, and Zambia have also engaged in the commercial production of ethanol (RFA, 2017; Timilsina & Shrestha,

2011). Malaysia is also starting to develop alternative energy sources to support its population and commercial energy demand due to the drastic growth in population and economy (Goh *et al.*, 2010; Mekhilef *et al.*, 2011). Various energy-related policies with great foresight and vision are formulated, such as the National Petroleum Policy (1975), National Energy Policy (1979), National Depletion Policy (1980), Fuel Diversification Policy (1981,1999), Electricity Supply Act (1990), Gas Supply Act (1993), Fifth Fuel Policy (2000), and Energy Commission Act (2001), as intensive efforts to safeguard the supply of energy from both depletable and renewable resources (Chua & Oh, 2010; Goh *et al.*, 2010).

In March 2006, the National Biofuel Policy was formulated and implemented in which biofuel is emphasized as one of the five energy sources for Malaysia, as well as enhancing the nation's prosperity and well-being (Chin & H'ng, 2013; Chin, 2011). This policy is in line with nation's Five-Fuel Diversification Policy where renewable energy is promoted as the fifth fuel along with fossil fuels and hydropower (Oh *et al.*, 2010; Sulaiman *et al.*, 2011). According to this plan, biodiesel, in particular, is to be produced from palm oil for local use and for export. However, the implementation of the whole biodiesel project has been put on hold indefinitely due to the current high price of refined, bleached and deodorized palm olein (Chin & H'ng, 2013).

Biomass has become the most attractive option for Malaysia since this country is gifted with natural resources, and it generates large quantity of lignocellulosic biomass from agriculture wastes, forest residues and municipal solid wastes (Goh *et al.*, 2010). However, bioethanol from lignocellulosic biomass is a relatively new idea in Malaysia and the development of lignocellulose-related technologies is also not very well-established in the world as stated by

Goh *et al.* (2010). Hence, through the National Biomass Strategy 2020, a new strategy was launched in 2011, in order to promote the biofuel by aiming to create higher value-added biomass economic activities that contribute towards Malaysia's gross national income (GNI) as well as creating high value jobs for the benefits of Malaysians. Under this strategy, the production of bioethanol is not only from oil palm but is also extended to include other lignocellulosic biomasses such as wood waste (Agensi Inovasi Malaysia, 2013; Chin & H'ng, 2013).

Another biomass utilisation programme, the National Biomass Strategy 2020, proposed a mandate of bioethanol blending of 10% in petrol fuel in Malaysia by 2020, which will generate a domestic demand for one million tonnes of bioethanol per annum in Malaysia by 2020 (Chin & H'ng, 2013). As a result, much attention has been focused on generating bioethanol from oil palm biomass and wood waste (Agensi Inovasi Malaysia, 2013; Chin & H'ng, 2013). Other lignocellulosic biomass such as sago wastes can also be utilised as substrate in ethanol production as this industrial waste is in abundance in Sarawak, Malaysia (Awg-Adeni *et al.*, 2012; Vincent *et al.*, 2015a; Vincent *et al.*, 2015b).

2.11 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, or commonly known as brewer's yeast or baker's yeast, is a unicellular eukaryotic microbe (Bamforth, 2005; Duina *et al.*, 2014). This yeast is globular-shaped (Figure 2.20A) and belongs to the kingdom of fungi which possesses plant-like cell walls but have other features more in common with animals (Alberts *et al.*, 2008; Kschischo

et al., 2016). Yeast has a cell wall made of chitin with no peptidoglycan (Bamforth, 2005). *S. cerevisiae* is considered as yeast with respect to the single-celled morphology and the impossibility of creating sporocarp, like other fungi do (Duina *et al.*, 2014).

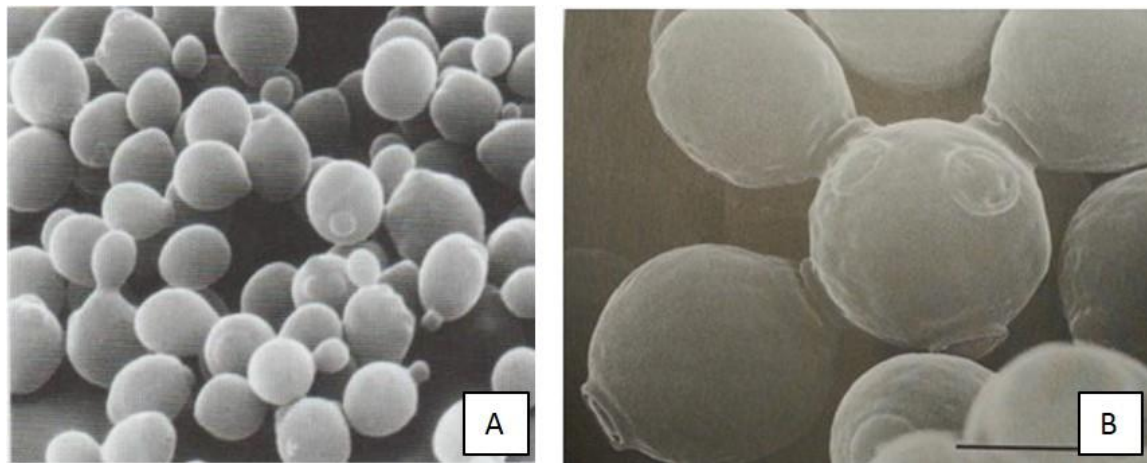


Figure 2.20: The yeast *S. cerevisiae* (A) A scanning electron micrograph of a cluster of the cell (Alberts *et al.*, 2008); (B) Yeast cell budding (Bamforth, 2005).

S. cerevisiae grows by budding (Figure 2.20B), which requires highly targeted cell growth and mechanisms for spatial coordination with nuclear division (Forsburg, 2005). Like all organisms, *S. cerevisiae* obtain their energy for synthesis, transport and reproduction purposes through cellular respiration which involves the oxidation of organic molecules. Some of the energy produced by this oxidation is stored in the chemical bonds of adenosine triphosphate (ATP). *S. cerevisiae* is categorized as facultative anaerobe, meaning that it is capable of both aerobic and anaerobic respiration (Petro, 2014). It is fairly simple in its nutritional requirement which requires a reduced carbon source, various minerals and a supply of nitrogen and vitamins (Bamforth, 2005).

Generally, *S. cerevisiae* preferentially ferments hexose sugars such as glucose and mannose, and it is not capable of fermenting pentose sugars such as xylose. It is because yeast does

not possess a functional xylose assimilation pathway (da Figueiredo Vilela *et al.*, 2015; Elbing *et al.*, 2004; Ha *et al.*, 2011). Glucose is the main carbon and energy sources for most yeasts, which is transported into cell through facilitated diffusion with the aid of at least 20 different transport proteins (Leandro *et al.*, 2009). Once inside the cell, through the Embden-Meyerhorf-Parnas (EMP) pathway, glucose is phosphorylated by hexokinase to glucose-6-phosphate and converted into pyruvate in a series of reactions, resulting in a net formation of 2 ATP (adenosine triphosphate) and 2 NADH (nicotinamide adenine dinucleotide) per sugar molecule (Bekatorou *et al.*, 2006).

In a fully respiratory metabolism of *S. cerevisiae*, pyruvate is decarboxylated through the pyruvate dehydrogenase and the resulting acetyl-CoA is subsequently oxidized to carbon dioxide in the tricarboxylic acid (TCA) cycle. This cycle plays an important role in yielding energy in the form of ATP, amino acid and lipid synthesis in yeast (Bekatorou *et al.*, 2006; Camarasa *et al.*, 2003). According to Bekatorou *et al.* (2006), when the concentrations of glucose are high enough, fermentation takes over respiration as enzymes used in respiration are repressed. During anaerobic fermentation, pyruvate is instead decarboxylated to acetaldehyde via the pyruvate decarboxylase and further reduced to ethanol by alcohol dehydrogenase (Rezaei *et al.*, 2015). *S. cerevisiae* prefers fermentation and decreases the level of TCA enzymes in order to balance the biosynthesis of proteins and the energy requirements of the cell (Camarasa *et al.*, 2003; Piškur *et al.*, 2006).

Traditionally, *S. cerevisiae* is an important cell factory for production of traditional foods, enzymes, and pharmaceuticals (Wang *et al.*, 2016). *S. cerevisiae* has been employed as a bioengineering platform in sugar- and starch-based ethanol production as well as in

lignocellulosic biorefineries (Aristidou, 2007; Reis *et al.*, 2013). *S. cerevisiae* is superior to bacteria, other yeasts, and filamentous fungi, due largely to its unique physiological characteristics (Tesfaw & Assefa, 2014). Some desirable properties of *S. cerevisiae* as an ethanol producer include fast growth rate, efficient glucose repression, efficient ethanol production and a tolerance for environment stresses such as high ethanol concentration and low oxygen levels, are fundamental to its industrial usefulness (Dombek and Ingram, 1987; Reis *et al.*, 2013). Because of these reasons, *S. cerevisiae* has been used in ethanol production from various types of feedstocks such as corn starch (Bialas *et al.*, 2014), banana peel waste (Parthiban *et al.*, 2011), sago hampas (Vincent *et al.*, 2015a; Vincent *et al.*, 2015b), tapioca sago waste (Subashini *et al.*, 2010) and sugarcane bagase (Wong & Sanggari, 2014).

2.12 Amylolytic Enzymes

Amylolytic enzymes are a group of hydrolases that can specifically cleave the O-glycosidic bonds in starch molecules (Kaur *et al.*, 2015). These enzymes are one of the great important families of enzymes in the field of biotechnology (Zar *et al.*, 2013). With the aid of amylolytic enzymes, starch molecules can be degraded into diverse products including dextrins and progressively smaller polymers composed of glucose via multistage processes, which included gelatinisation, liquefaction and saccharification (Ramesh & Murty, 2014; Ruiz *et al.*, 2011; Zar *et al.*, 2013).

The first amylase enzyme was discovered by Kirchhoff in 1811 (Gupta *et al.*, 2003). Amylases can be divided into two categories, which are endoamylases and exoamylases (Zar

et al., 2013). In the first liquefying steps, amylases are used to break the large chains of starch into much smaller, simpler sugars (Provost *et al.*, 2016). Subsequently, saccharification has taken place to break down the large chain of starch into very small fragments such as glucose, maltose and maltotriose (Kim *et al.*, 2004; Provost *et al.*, 2016; Ramesh & Murty, 2014). For successive starch hydrolysis, α -amylase and glucoamylase are required as both α -1,4 and α -1,6-debranching hydrolases (Aydemir *et al.*, 2014; Li *et al.*, 2010).

α -amylases (endo-1,4- α -D-glucanglucohydrolases; EC. 3.2.1.1) are microbial enzymes that derived from several fungi, yeasts, bacteria and actinomycetes. It is widely used in industrial processes such as brewing, baking, textiles, pharmaceuticals, starch processing and detergents (Demirkan, 2010; Gupta *et al.*, 2003; Singh *et al.*, 2016). α -amylases belong to the glycoside hydrolase family 13 (GH13) and cleave the endo-hydrolysis of 1,4- α -D-glycosidic linkages between adjacent 1,4- α -linked glucose units in the amylose (Cao *et al.*, 2015). The enzyme acts on starch in a random manner, liberating in formation of low molecular weight reducing groups such as maltose, glucose and maltotriose (Demirkan, 2010; Parbat & Singhal, 2011; Raul *et al.*, 2014; Singh *et al.*, 2016).

Glucoamylases (exo-1,4- α -D-glucanglucanohydrolases or β -amylase; EC 3.2.1.3) are one of the oldest and widely used enzymes in food industry, especially in the enzymatic production of glucose and fructose from starch (Bhatti *et al.*, 2007; Hostinová & Gašperík, 2010; Lawal *et al.*, 2014; Ritslaid *et al.*, 2010). These extracellular and microbial enzymes are starch hydrolysing enzyme capable of catalysing α -1,4-glucosidic linkages to release the inverted β -D-glucose units from the non-reducing ends of starch molecules and to hydrolyse α -1,6-

glucosidic bonds on amylopectin branching points, also resulting in glucose as end product (Hostinová & Gašperík, 2010; Nahar *et al.*, 2008; Slivinski *et al.*, 2011). This enzyme can degrade both amylose and amylopectin and promote an almost complete starch hydrolysis into glucose (Nahar *et al.*, 2008; Ramesh & Murty, 2014; Slivinski *et al.*, 2011).

2.13 Cellulolytic Enzyme

In lignocellulosic ethanol production, hydrolysis processes demand cellulolytic enzymes to perform the degradation of cellulosic materials into fermentable sugars (Agostinho *et al.*, 2015; Hsieh *et al.*, 2014). Kuhad *et al.* (2011) stated that cellulases have been commercially available since 30 years ago, and these enzymes have been targets for both academic and industrial research. Cellulase is a family of at least 3 groups of enzymes, which are endo-(1,4)- β -D-glucanase (EC 3.2.1.4), exo-(1,4)- β -D-glucanase (EC 3.2.1.91) and β -glucosidases (EC 3.2.1.21). These inducible enzymes are synthesized by a large diversity of microorganisms that have cellulolytic abilities including both fungi and bacteria as listed in Table 2.9.

Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications (Kuhad *et al.*, 2011). Cellulase is constructed of independently folding, structurally and functionally specialised units called domains or modules (Kuhad *et al.*, 2011; Maki *et al.*, 2008). A typical cellulase consists of cellulose binding domains and catalytic domains that are separated by a flexible peptide linker which acts as a flexible arm connecting the two parts together (Kuhad *et al.*, 2011; Lakhundi *et al.*, 2015; Zhang *et al.*, 2015).

Table 2.9: Microorganisms having cellulolytic abilities (Kuhad *et al.*, 2011).

Fungi	Soft rot fungi <i>Aspergillus niger</i> ; <i>A. nidulans</i> ; <i>A. oryzae</i> ; <i>A. terreus</i> ; <i>Fusarium solani</i> ; <i>F. oxysporum</i> ; <i>Humicola insolens</i> ; <i>H. grisea</i> ; <i>Melanocarpus albomyces</i> ; <i>Penicillium brasilianum</i> ; <i>P. occitanis</i> ; <i>P. decumbans</i> ; <i>Trichoderma ressei</i> ; <i>T. longibrachiatum</i> ; <i>T. harzianum</i> ; <i>Chaetomium cellulyticum</i> ; <i>C. thermophilum</i> ; <i>Neurospora crassa</i> ; <i>P. fumigatum</i> ; <i>Thermoascus aurantiacus</i> ; <i>Mucor circinelloides</i> ; <i>P. janthinellum</i> ; <i>Paecilomyces inflatus</i> ; <i>P. echinulatum</i> ; <i>Trichoderma atroviride</i>
	Brown rot fungi <i>Coniophora puteana</i> ; <i>Lanzites trabeum</i> ; <i>Poria placenta</i> ; <i>Tyromyces palustris</i> ; <i>Fomitopsis</i> sp.
	White rot fungi <i>Phanerochaete chrysosporium</i> ; <i>Sporotrichum thermophile</i> ; <i>Trametes versicolor</i> ; <i>Agaricus arvensis</i> ; <i>Pleurotus ostreatus</i> ; <i>Phlebia gigantea</i>
	Aerobic bacteria <i>Acinetobacter junii</i> ; <i>A. amitatus</i> ; <i>Acidothermus cellulolyticus</i> ; <i>Anoxybacillus</i> sp.; <i>Bacillus subtilis</i> ; <i>B. pumilus</i> ; <i>B. amyloliquefaciens</i> ; <i>B. licheniformis</i> ; <i>B. circulans</i> ; <i>B. flexus</i> ; <i>Bacteriodes</i> sp.; <i>Cellulomonas biazotea</i> ; <i>Cellvibrio gilvus</i> ; <i>Eubacterium cellulosolvens</i> ; <i>Geobacillus</i> sp.; <i>Microbispora bisporea</i> ; <i>Paenibacillus curdlanolyticus</i> ; <i>Pseudomonas cellulose</i> ; <i>Salinivibrio</i> sp.; <i>Rhodothermus marinus</i>
Bacteria	Anaerobic bacteria <i>Acetivibrio cellulolyticus</i> ; <i>Butyrivibrio fibrisolvens</i> ; <i>Clostridium thermocellum</i> ; <i>C. cellulolyticum</i> ; <i>C. acetobutylicum</i> ; <i>C. papyrosolvens</i> ; <i>Fibrobacter succinogenes</i> ; <i>Ruminococcus albus</i>
	<i>Cellulomonas fimi</i> ; <i>C. bioazotea</i> ; <i>C. uda</i> ; <i>Streptomyces drozdowiczii</i> ; <i>S. lividans</i> ; <i>Thermomonospora fusca</i> ; <i>T. curvata</i>
Actinomycetes	

The components of cellulase system are classified into three major groups according to the mode of catalytic action and structural properties (Zhang & Zhang, 2013). These are generically termed as endoglucanases, exoglucanases and β -glucosidases. Enzymatic hydrolysis by these three enzyme groups occur simultaneously (Figure 2.21) to disrupt the structure at the solid-liquid interaction making the individual fibre available for hydrolysis (Lakhundi *et al.*, 2015). Processive cellulases, both endoglucanase and exoglucanase, perform the synergistic primary hydrolysis on the surface of crystalline cellulose, where endo-enzymes generate new reducing and non-reducing chain ends for the exo-enzyme to produce soluble sugars (Horn *et al.*, 2012; Lakhundi *et al.*, 2015; Zheng & Ding, 2013). β -

Glucosidases then perform the secondary hydrolysis on these soluble cellodextrins or cellobiose to glucose (Lakhundi *et al.*, 2015; Quiroz-Castañeda & Folch-Mallol, 2013).

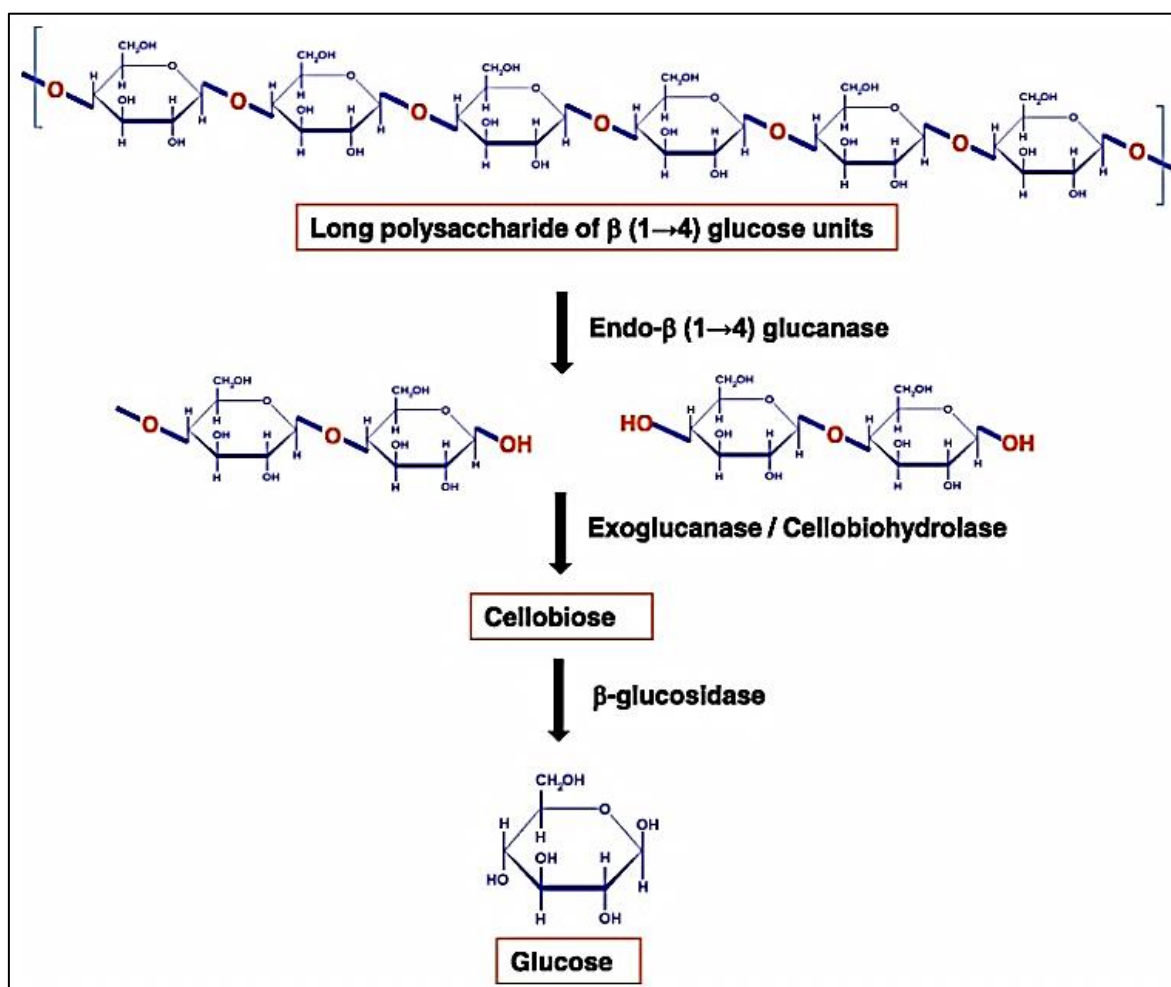


Figure 2.21: The mode of mechanisms of cellulolytic enzymes (Lakhundi *et al.*, 2015).

Endo-(1,4)- β -D-glucanases (Endoglucanases; EC 3.2.1.4) are monomeric enzymes with a molecular weight that ranges from 22 to 45 kDa (Juturu & Wu, 2014; Quiroz-Castañeda & Folch-Mallol, 2013). Endoglucanases produce random internal cuts within the amorphous region in the cellulose fibres with optimum temperature 50-70 °C and pH of 4-5, yielding cello-oligosaccharides of various length and thereby creating new free chain-ends (Lakhundi *et al.*, 2015; Quiroz-Castañeda & Folch-Mallol, 2013). Thus, endoglucanases activities can

be assayed using excellent soluble amorphous cellulose substrates which is carboxymethylcellulose (CMC). It is because of this assay that endoglucanases are also termed as carboxymethylcellulase (CMCase) (Zhang & Zhang, 2013; Quiroz-Castañeda & Folch-Mallol, 2013).

Exo-(1,4)- β -D-glucanases (Exoglucanases; EC 3.2.1.74), also known as cellobiohydrolases (CBHs; EC 3.2.1.91), are monomeric proteins that have molecular weights of between 50 and 65 kDa (Kuhad *et al.*, 2016; Quiroz-Castañeda & Folch-Mallol, 2013; Zheng & Ding, 2013). These enzymes are able to hydrolyse crystalline cellulose and the optimum conditions are at 37 to 60 °C and at pH of 4 to 5. These isoenzymes work specifically on the ends of cellulose by involving two main types of exoglucanase that acts in a processive manner on the reducing and non-reducing ends of cellulose, liberating cellobiose molecules as main product. Exoglucanases are also found in multiple copies, at times accounting for 40.0%-70.0% of the total component of the cellulase system. Furthermore, crystalline cellulose is documented as an excellent substrate for exoglucanase activity assay due to its low DP and relatively low accessibility (Quiroz-Castañeda & Folch-Mallol, 2013; Zheng & Ding, 2013).

β -glucosidases (EC 3.2.1.21) can be monomeric or exist as homo-oligomers that has a larger range of molecular weights of between 35 and 640 kDa (Quiroz-Castañeda & Folch-Mallol, 2013; Zheng & Ding, 2013). According to Singhania *et al.* (2013), these enzymes are the key enzymes component present in cellulase and complete the final step of cellulose hydrolysis by splitting soluble cellobiose and cellodextrins to produce glucose in aqueous phase. This reaction is optimal under a wide pH ranges from 3.5 to 5.5 and in temperature ranges from 45 to 75 °C (Quiroz-Castañeda & Folch-Mallol, 2013). The hydrolysis of

cellobiose and cellodextrins is highly important in order to eliminate cellobiose inhibitor (Teugjas & Vāļamā, 2013). The activities of β -glucosidases can be assayed using cellobiose, which is not hydrolysed by endoglucanase and exoglucanase (Quiroz-Castañeda & Folch-Mallol, 2013).

2.14 Simultaneous Saccharification and Fermentation (SSF)

Lignocellulosic biomass can be enzymatically hydrolysed and fermented in different process configurations. Two most common are simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) (Sarkar *et al.*, 2012; Yan *et al.*, 2015). SSF is the concept of process that combines the enzymatic saccharification of the lignocellulosic biomass and fermentation of resultant sugar to ethanol in the same vessel, at the same time (Ask *et al.*, 2012). The initial concept of conducting the enzymatic saccharification and fermentation simultaneously was first described in 1976 by Gauss and colleagues (Ask *et al.*, 2012; Ruiz *et al.*, 2012). In this process, the enzymes and fermenting microorganism are added simultaneously at the beginning of the fermentation process. The fermentable sugars from the enzymatic hydrolysis of lignocellulosic biomass is simultaneously metabolised by fermenting microorganism in the broth, and then convert the fermentable sugars to produce ethanol (Triwahyuni *et al.*, 2015).

SSF has been shown to be superior to SHF in terms of overall ethanol yield (Ruiz *et al.*, 2012). As reported by Sarkar *et al.* (2012), SSF offers the advantage of removing the sugar accumulation inhibition that is minimizing the yield of ethanol in SHF process. It is because

the enzymes activities can be inhibited with the presence of sugars (Ruiz *et al.*, 2012). Thereby, the fast conversion of glucose into ethanol can reduce the enzyme inhibition, while it lends to time consuming and consequence of the increases in ethanol productivity (Ask *et al.*, 2012; Sarkar *et al.*, 2012). Enzyme sterile conditions requirements in SSF are lowered as glucose is converted immediately into ethanol (Kroumov *et al.*, 2015). The potential loss of fermentable sugars such as glucose and xylose can be minimized by using the complete slurry without separation of the supernatant constituents (Ask *et al.*, 2012; Triwahyuni *et al.*, 2015). SSF is cost effective, especially in reducing equipment costs due to the hydrolysis and fermentation of substrates in a single reactor (Ask *et al.*, 2012; Ruiz *et al.*, 2012).

One major bottleneck with the SSF process is that the conditions for the enzymatic saccharification and the fermentation have to be the same, meaning it is conducted at temperatures inferior to optimum of the cellulolytic enzymes (Ask *et al.*, 2012). For the enzymatic saccharification step, the optimum temperature of cellulolytic enzymes is around 50 °C, whereas most fermenting microorganisms has an optimum temperature ranging between 30 to 37 °C (Yan *et al.*, 2015). As suggested by Moreno *et al.* (2013), the use of thermotolerant microorganisms offer a number of potential advantages such as the shorter time required of SSF, reduced cooling costs, and better saccharification and ethanol yields.

CHAPTER 3

BIOETHANOL PRODUCTION FROM SAGO EFFLUENT HYDROLYSATE USING *Saccharomyces cerevisiae* VIA SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

3.1 Introduction

The cost of various fossil fuels in the world market such as oil, coal and natural gas have increased with the growing demand to produce fuel, electricity and other goods (Sarkar *et al.*, 2012). These growing demands is also causing security issues that negatively impact global economy, disruptions in supplies and environmental problems such as global warming and pollutions (Singh *et al.*, 2015). In order to eliminate these negative impacts, the development of renewable and sustainable energy resources such as wind, solar, biomass and geothermal are spawning increasing public and scientific attentions (Aditiya *et al.*, 2016; Bhatia *et al.*, 2012; Shah & Sen, 2011). Among the alternative energies, bio-renewable fuel is one of the fastest growing as it contributes significantly the reduction of harmful emissions and reducing the world's dependence on fossil fuels (Colbran & Eide, 2008; Ji & Long, 2016; Naik *et al.*, 2010).

Bio-renewable fuel or biofuel, is available in many forms such as bioethanol, biodiesel and biogas. It is manufactured from renewable material such as biomass resources (Aditiya *et al.*, 2016; Chin & H'ng, 2013). Of the many examples of biofuel, bioethanol is widely regarded as the most promising and sustainable liquid biofuel due to various advantageous properties (Singh *et al.*, 2012; Vincent *et al.*, 2015a). It is notably a clean-burning bio-

renewable energy that is biodegradable and environmentally friendly with a high octane number, broad flammability limits, high flame speed and vaporization heats. Accordingly, bioethanol has the ability to reduce emissions in spark-ignition engines which enables it to compete with fossil fuels in terms of efficiency (Adekunle *et al.*, 2016; Chin & H'ng, 2013).

Bioethanol is made from either conventional or advanced biofuel technologies, depending on the state of sugars polymerization (Chin & H'ng, 2013). A variety of carbohydrates can be utilised for fermentation, from simple sugars including glucose and sucrose, to more complex carbohydrates as well as carbohydrate-rich lignocellulosic biomass (Chin & H'ng, 2013; Ibeto *et al.*, 2011; Wong & Sanggari, 2014). Examples of carbohydrate source that have been studied are sugarcane molasses (Behera *et al.*, 2012) and office wastes (Vincent *et al.* 2014). Starchy materials and effluent generated from starch processing are also abundant and renewable feedstock available for second-generation bioethanol that do not compete with food production and animal feed (Kusmiyati, 2014; Limayem & Ricke, 2012; Verma *et al.*, 2000). In particular, the utilisation of free carbon available from discharges of starchy wastewater helps recover available energy as well as purify the effluent (Limayem & Ricke, 2012; Ritslaid *et al.*, 2010).

Starch from sago palm (*Metroxylon sagu*), also known as 'rumbia' by the locals, has long been a staple food as well as a dietary supplement (Awg-Adeni *et al.*, 2010; Karim *et al.*, 2008). The sago palm is considered as an extremely important socioeconomic entity due to its economic competitiveness in yield and price compared to other crops (Awg-Adeni *et al.*, 2010). Approximately 90% of the sago starch is produced from the biggest sago starch exporter in the world, the State of Sarawak, Malaysia (Chong *et al.*, 2014). The negative

aspect is that sago extractions generate huge amount of starchy wastewater, often termed as sago effluent. This effluent has an obnoxious odour, irritating colour, low pH, high BOD and COD content (Ramesh *et al.*, 2013).

Furthermore, allowing a significant amount of sago effluent into the aquatic ecosystems without proper treatment causes serious contamination due to the slow degradation rate of cellulosic fibrous materials in the effluent (Abd-Aziz, 2002; Diong *et al.*, 2016; Vincent *et al.*, 2015a). Most importantly, due to incomplete extraction practices, a considerable amount of sago starch was found to remain in the sago effluent (Bujang, 2014; Hasyim *et al.*, 2011). This biopolymer has the potential to be a major substrate for the production of ethanol (Aydemir *et al.*, 2014; Rebroš *et al.*, 2009; Ritslaid *et al.*, 2011). In recent years, studies have been conducted to reduce environmental impacts and to recover the energy lost in sago wastewater by utilising sago liquid waste for fermentative ethanol production (Anwar *et al.*, 2014; Subashini *et al.*, 2011). For example, Awg-Adeni *et al.* (2012) and Vincent *et al.* (2015a) have studied the production of bioethanol from the solid portion (sago hampas) of sago effluent under different processes.

In the present study, bioethanol production was performed via simultaneous saccharification and fermentation (SSF) on the liquid portion of sago effluent. This process is one of the most promising platforms for the bioconversion of lignocellulosic biomass into ethanol (Tom ás-Pejó *et al.*, 2009; Vincent *et al.*, 2014). Prior to ethanol fermentation, sago starch was hydrolysed via saccharification using amylolytic enzymes in the presence of fermenting microorganisms (Awg-Adeni *et al.*, 2012; Ritslaid *et al.*, 2010). Presently, the best-known fermenting microorganisms are the yeast, *Saccharomyces cerevisiae*, and the bacterium,

Zymomonas mobilis, as both have very high ethanol yields (Jessen & Orlygsson, 2012; Kang *et al.*, 2014; Vincent *et al.*, 2015a). However, *S. cerevisiae* was preferred for fermentative synthesis of ethanol as it is the safest and most effective (Aristidou, 2007; Vincent *et al.*, 2015a).

3.2 Materials and Methods

3.2.1 Experimental Setup

An overview of the experimental setup is shown in Figure 3.1. All experiments including analytical methods were performed in triplicates (n=3).

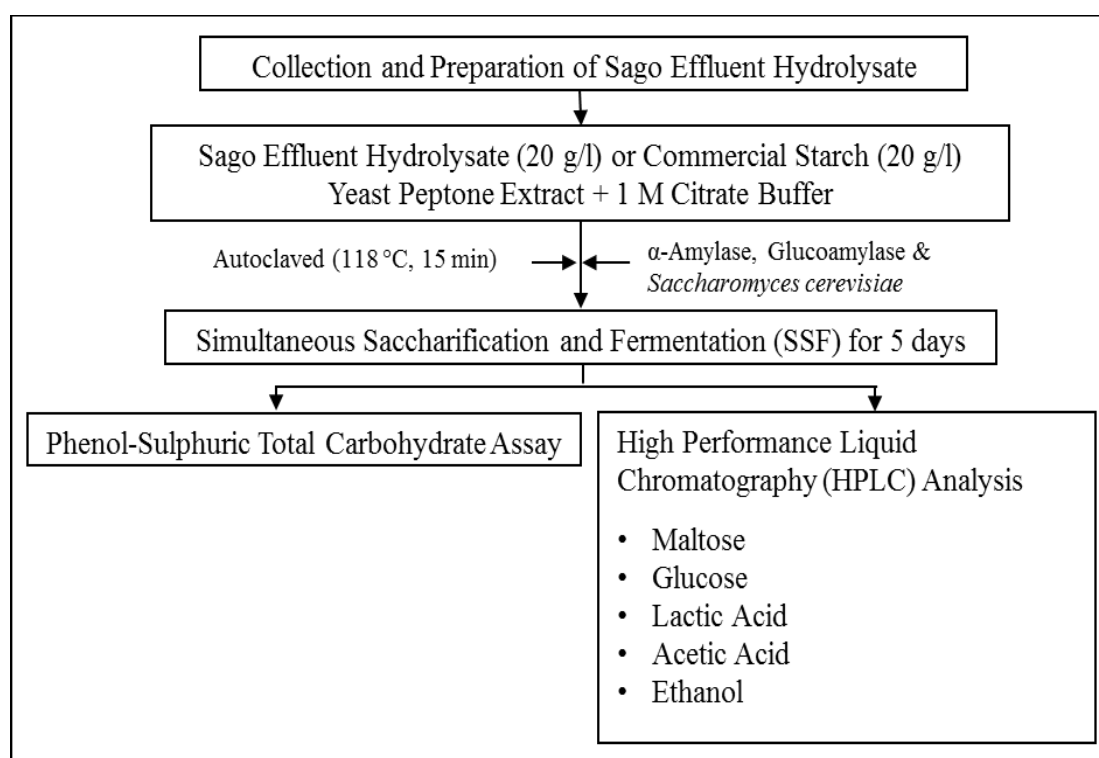


Figure 3.1: Flow chart outlining the steps for simultaneous saccharification and fermentation (SSF) of different feedstock loadings with amylolytic enzymes and the fermentative synthesis of ethanol of *S. cerevisiae*.

3.2.2 Collection and Preparation of Sago Effluent Hydrolysate

Sago effluent was retrieved from the Herdsen Sago Mill in Pusa, Sarawak. The sago effluent was filtered through a 710 µm stainless steel filter to eliminate large solid particles and sago hampas to obtain only the sago effluent hydrolysate that was used in this study.

3.2.3 Glycerol Stocks and Culture Preparation

Saccharomyces cerevisiae ATCC 24859 used in this study was obtained from the American Type Culture Collection. The yeast culture was revived in sterile Yeast Malt (YM) broth (HiMedia, India) by shaking (NB-101MT Multi Shaker, N-Biotek, Korea) at 120 rpm overnight at ambient temperature. For long term storage, the stock culture was prepared by mixing culture medium with supplementation of 20% (v/v) glycerol and aliquots of 2.0 ml were stored at -20 °C in an ultralow-temperature freezer (SJC203 Refrigerator, Sharp, Malaysia).

S. cerevisiae culture inoculum for SSF was prepared by growing an aliquot of the stock culture in 150 ml sterile YM broth in a 250 ml conical flask with constant agitation at 120 rpm overnight at ambient temperature. Next, the culture medium was transferred into 50 ml of Falcon tubes in equal volumes and centrifuged (BK-1032J Low Speed Centrifuge, Biobase, China) at 3,240 x g for 6 min to harvest the yeast cells (Vincent *et al.*, 2011). The supernatant was discarded and the cell pellets transferred into fermentation media.

3.2.4 Simultaneous Saccharification and Fermentation (SSF)

Saccharification and fermentation of sago effluent hydrolysate were performed simultaneously in the batch culture by using amylases and *S. cerevisiae* to investigate the

potential of sago effluent hydrolysate in producing ethanol. SSF was carried out in 500 ml Schott bottles containing 200 ml of batch culture media in 0.05 M citrate buffer. Sago effluent hydrolysate was supplemented with 3.0 g/l bacterial peptone (Bendosen Laboratory Chemicals, Malaysia) and 1.5 g/l yeast extract (Bacto, USA). The prepared batch culture media was sterilised in an autoclave (Model No. 25X Electric Pressure Steam Sterilizer, All American, United State) to provide sterility and to gelatinise the starch. The starch concentration in sago effluent hydrolysate was determined through the phenol-sulphuric acid assay. A commercial starch (Bendosen Laboratory Chemicals, Malaysia) was used as the experimental control. After the temperature of batch culture media has reduced to 60 °C, amylases (Sumson, China) were added and the media were left to cool down to 37 °C by taking one hour before being aseptically inoculated with *S. cerevisiae*. The batch cultures were then agitated at 130 rpm at ambient temperature for five days.

3.2.5 Sample Collection

Aliquots of 1.5 ml of samples were taken aseptically at predetermined time intervals: 0, 6, 12, 18, 24, 30, 36, 48, 72, 96, and 120 h. The samples were first centrifuged (*WiseSpin*® CF-10 High-Performance Microcentrifuge Set, Daihan Scientific, Korea) at 10,125 x g for 5 min and the supernatants were filtered through a 0.45 µm nylon syringe filter (Whatman, NJ, USA). Then, the prepared samples were stored in 1.50 ml microcentrifuge tubes at -20 °C prior to analysis.

3.2.6 Phenol-Sulphuric Total Carbohydrate Assay

Phenol-sulphuric assay (Crawford & Pometto, 1988) is a simple colorimetric method to determine total carbohydrate in a sample solution. The assay was performed by recording

the optical density of the filtered samples at 490 nm (OD₄₉₀) using a Unicam dual-beam recording spectrophotometer (SP-880 Visible Spectrophotometer, Metertech, Taiwan). The equivalent sugar concentration (g/l) was determined based on a standard glucose concentration curve that was generated prior to the assay (Vincent *et al.*, 2014).

3.2.7 High Performance Liquid Chromatography (HPLC) Analysis

The filtered samples were analysed using a High Performance Liquid Chromatography (LC-20A HPLC, Shimadzu, Japan) to quantify the concentration of residual sugars (maltose and glucose) and fermentation products (ethanol, acetic acid and lactic acid). The HPLC system was equipped with a Prominence column oven (CTO-20A, Shimadzu, Japan), refractive index detector (RID-10A, Shimadzu, Japan), Prominence communication bus module (CBM-20A, Shimadzu, Japan) and a computer controller. The separation of samples (20 µl injection volume per sample) was performed on a 150 x 7.8 mm Aminex HPX-87H column (Bio-Rad Chemical Division, California, USA) with 0.005 M H₂SO₄ as the mobile phase at a flow rate of 0.8 ml/min, with the column temperature set at 65 °C (Vincent *et al.*, 2011). The concentrations of residual sugar and fermentation products (g/l) were determined based on a standard concentrations curves that were generated prior to the sample injections according to retention times as below: -

Table 3.1: The retention times of components through HPLC analysis.

Components	Retention Time (min)
Maltose/cellobiose	≈ 3.195
Glucose	≈ 3.895
Xylose	≈ 4.162
Arabinose	≈ 4.859
Lactic Acid	≈ 5.385
Acetic Acid	≈ 6.062
Ethanol	≈ 8.369

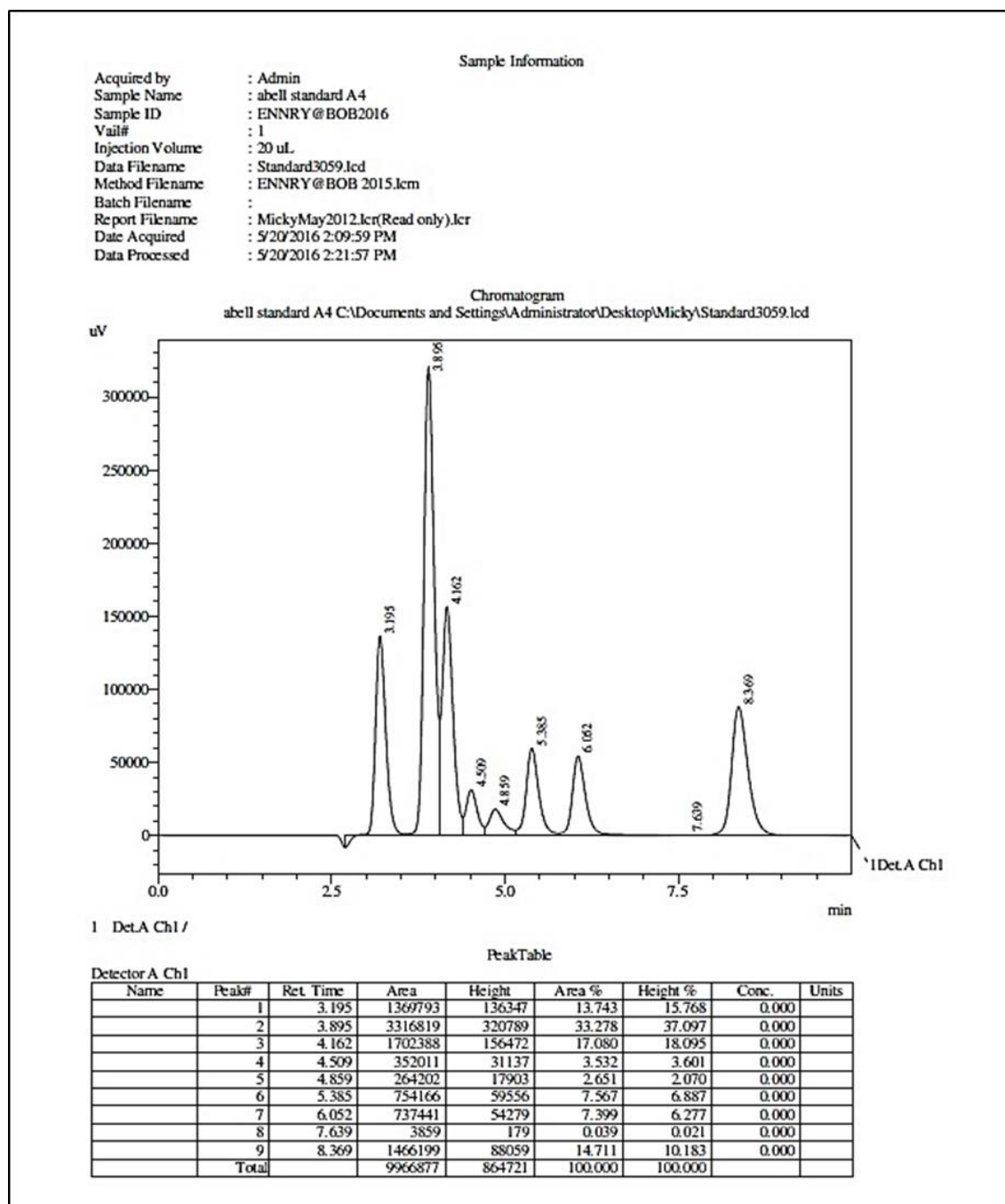


Figure 3.2: The chromatogram of High Performance Liquid Chromatography

3.2.8 Statistical Analysis

All collected data were plotted into graphs on concentrations of total carbohydrates, maltose, glucose, lactic acid, acetic acid and ethanol. Error bars were determined based on the

standard deviation from the mean value of triplicate experiments. One-Way Univariate ANOVA (F-test) for significant differences was performed using IBM® SPSS® Statistics Software version 22 for all final data set for comparison of the concentration of two different substrates. A p-value greater than 0.05 was considered statistically insignificant.

3.3 Results and Discussion

Sago effluent generated from starch producing operations such as sago mill is an inexpensive and abundant substrate for starch-derived bioethanol that has many advantages in both energetic and environmental points of view (Olofsson *et al.*, 2008; Yunus *et al.*, 2014). This carbohydrate-rich effluent is usually discarded into nearby stream and resulted in serious contamination (Awg-Adeni *et al.*, 2010). In this study, simultaneous saccharification and fermentation (SSF) was selected as the method for bioethanol production from sago liquid residue (sago effluent hydrolysate) as it was shown to be superior to other methods such as separate hydrolysis and fermentation (SHF). In SSF, enzymatic hydrolysis and fermentation take place simultaneously in same vessel. This method has the advantages of avoiding end-product inhibition of the hydrolysis process, minimizing the risk of contamination and reducing cost. Consequently, the saccharification rate and ethanol yield is considerably higher in SSF than other methods (Ohgren *et al.*, 2007; Olofsson *et al.*, 2008; Vincent *et al.*, 2015a).

Phenol-sulphuric acid assay was carried out to determine the concentration of total carbohydrates in the sample of sago effluent hydrolysate obtained. Initial analyses recorded 20.00 ± 0.22 g/l of total carbohydrate in the sago effluent hydrolysate, which was almost

similar to a previous report by Bujang (2014). Commercial starch was used as the control to gauge the potential of sago effluent hydrolysate as the substrate in the production of ethanol via SSF. All fermentation broths were autoclaved for the purpose of sterilisation as well as gelatinisation, where the crystalline organisation of starch decomposes to form amorphous regions and water molecules disrupt the hydrogen bonds within and between starch molecules in the presence of heat (Beninca *et al.*, 2008; Ritslaid *et al.*, 2010). This molecular disorder plays a pivotal role as gelatinised starch is more easily hydrolysed to yield individual glucose molecules via enzymatic activity (Safitri & Wiyono, 2013; Ritslaid *et al.*, 2010).

Upon gelatinisation, α -amylase (12.5 U/ml) and glucoamylase (25.0 U/ml) were introduced into the sample to act as catalysts. These enzymes play a significant role in the degradation of starch by hydrolysing the glycosidic bonds between adjacent glucose units of the polymer (Kim *et al.*, 2004; Nguyen *et al.*, 2002; Ominyi Matthias, 2013). α -Amylase attacks the second linkage of the straight chain of starch molecule from the non-reducing terminal and result in the splitting off of two glucose units and maltose (Li *et al.*, 2010; Ominyi Matthias, 2013). To a lesser extent, glucoamylase hydrolyses both α -1,4- and α -1,6-glycosidic bonds, releasing glucose as the final products (Ominyi Matthias, 2013). Hence, α -amylase and glucoamylase were supplemented together for successful saccharification of starch into fermentable sugars. Fermentation of enzymatic saccharification products was further achieved by employing the yeast *S. cerevisiae*.

In order to evaluate the suitability of sago effluent hydrolysate as a feedstock for bioethanol production, fermented samples collected at predetermined time points were measured for

total carbohydrates residues. In both experiment and control, the initial total carbohydrates were 19.00 g/l as buffered with 50 mM citrate buffer to provide optimum pH in fermentation broth. As shown in Figure 3.3, the experiment and control exhibited almost identical patterns in the changes in total carbohydrates concentrations over time. The total carbohydrates in both systems decreased steeply within the first 6 hours of SSF and remained virtually constant thereafter, with those of sago effluent hydrolysate (1.98 g/l) slightly higher than commercial starch (0.83 g/l). The results from phenol-sulphuric acid assay quantified 1.57 g/l of residual carbohydrates at the end of fermentation, indicating that 91.70% of total carbohydrate was being utilised for fermentation. Meanwhile, 96.80% of total carbohydrates in commercial starch was being used up at the end of fermentation, showing a very close utilisation of carbohydrate in both systems.

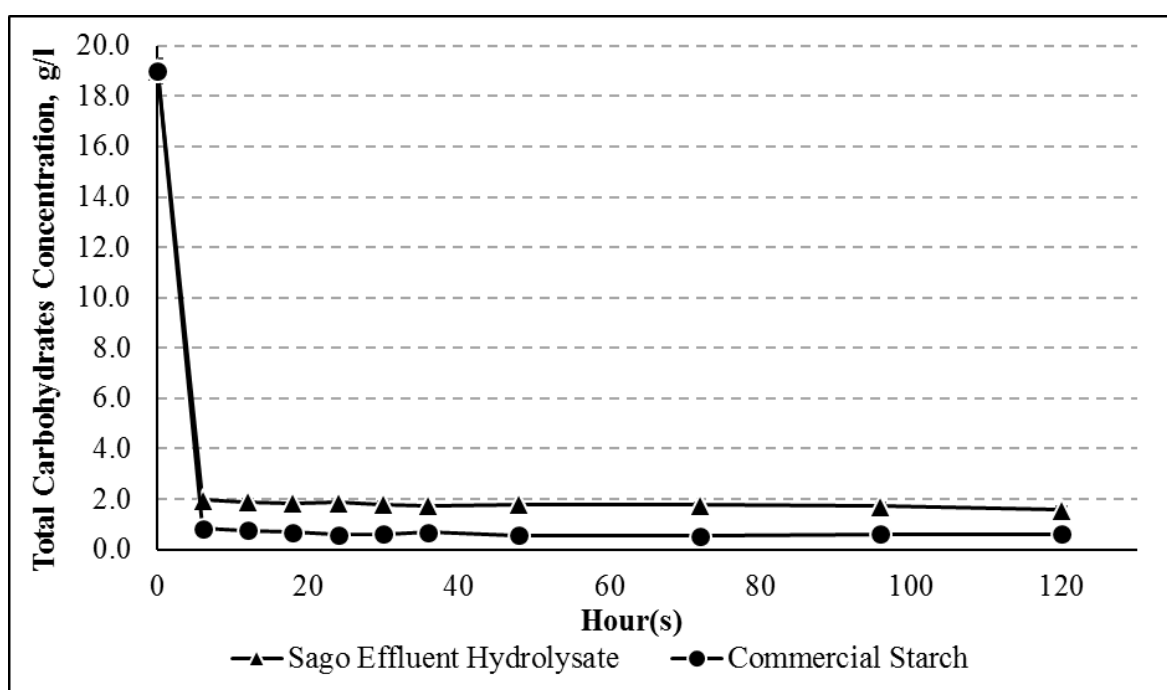


Figure 3.3: Time course of total carbohydrate, as determined via the phenol-sulphuric acid assay. [Note: The data points represent the average of three independent experiments (n=3).]

To complement the residual carbohydrate analysis, glucose and maltose were quantified using HPLC. These sugars are the main end products from the hydrolysis of starch polymers. Glucose was detected immediately at 0 h, at 14.50 g/l and 17.66 g/l (Figure 3.4) in sago effluent hydrolysate and commercial starch, respectively. These concentrations decreased drastically within 6 h, followed by glucose depletion at 18 h, indicating the glucose molecules released from starch was promptly utilised by *S. cerevisiae*. Studies showed that the hydrolysis process in SSF resulted in better conversion yields when high glucose concentration are present at the initial stage of fermentation. According to Awg-Adeni *et al.* (2012), this is due to preferential hydrolysis of the amorphous region at the early stage of the saccharification.

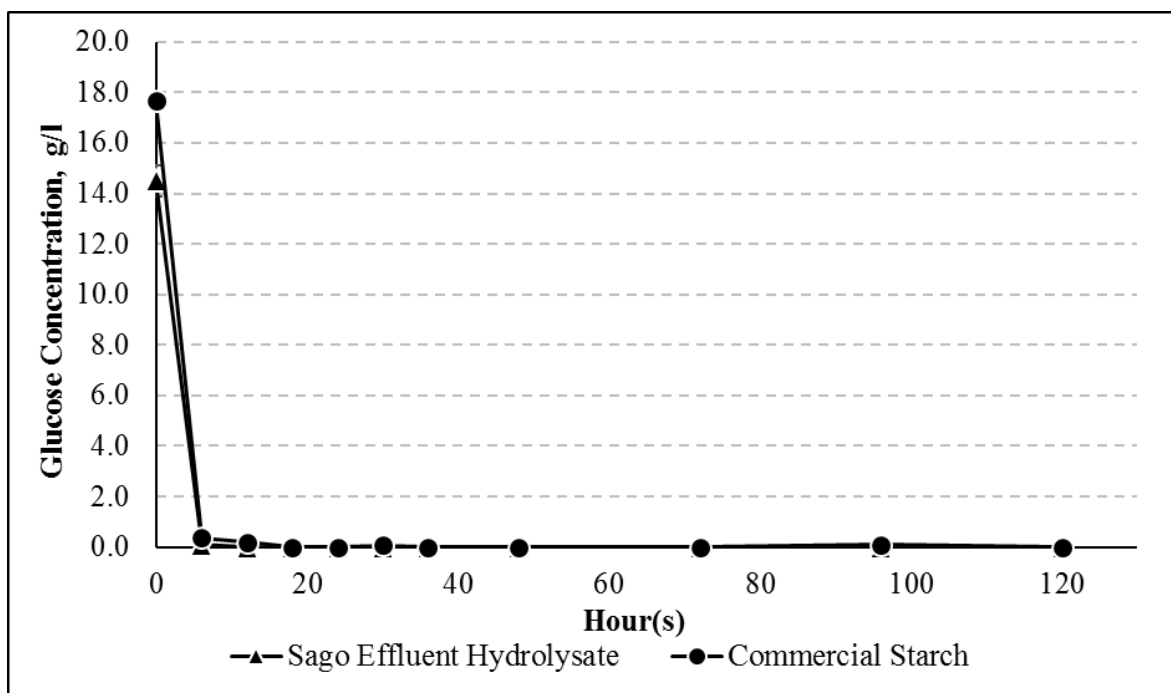


Figure 3.4: Time course of glucose concentration, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

Figure 3.5 shows the time course of maltose concentration during the SSF with sago effluent hydrolysate and commercial starch. The initial amount of maltose was approximately 1.00 g/l in both sago effluent hydrolysate and commercial starch. After 6 h, no more maltose was detected in both substrate loadings, indicating complete hydrolysis was achieved by the amylolytic enzymes involved and maltose was completely converted into glucose.

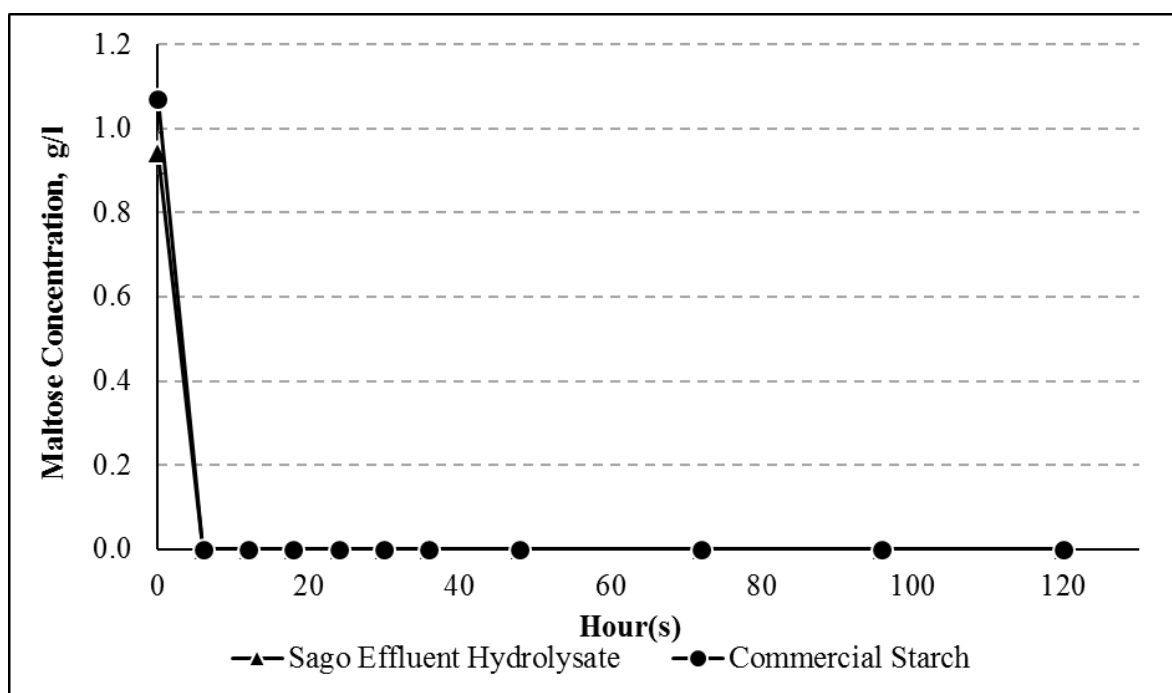


Figure 3.5: Time course of maltose concentration, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

During the first 6 hours of fermentation, ethanol production of sago effluent hydrolysate showed a steep increase to 7.75 g/l (76.68% TEY). This trend continued until 12 h with a peak production of 8.39 g/l or 84.89% TEY. This value is almost identical to the yield in commercial starch, which was 8.88 g/l or 91.49% TEY, at the same time point. Both substrates also showed slow decrease in ethanol yield over the course of the experiment after a steep production of ethanol in the first 6 hours, with a peak at 12 h of SSF. This very similar

pattern of ethanol production over time indicates that sago effluent hydrolysate is comparable to commercial starch as a source for ethanol production via SSF.

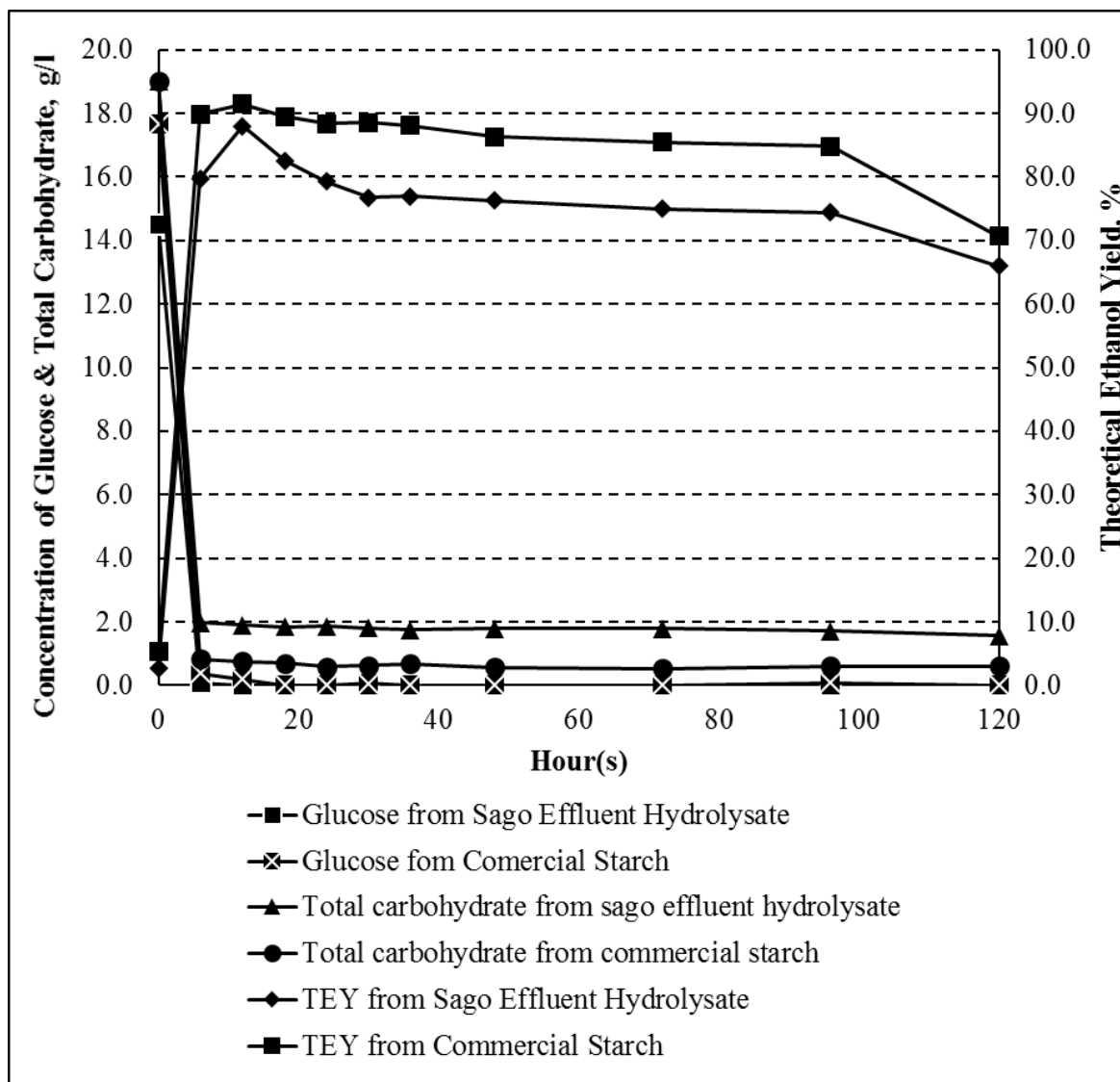


Figure 3.6: Time course of theoretical ethanol yield, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

From the results shown in Figure 3.6, ethanol production is shown to be correlated to the concentration of fermentable sugars in the fermentation media. Although both substrate loadings had the same total carbohydrate concentrations at 0 h, a significant difference is

observed in the concentration of residual carbohydrate at the end of fermentation period (120 h). The residual carbohydrates in commercial starch (0.61 g/l) were slightly lower than in sago effluent hydrolysate (1.57 g/l). The explanation for a higher ethanol production in the broth containing commercial starch is that it contained more fermentable sugars to be converted into ethanol by the yeast cells. On the other hand, it was noted that slightly higher ethanol yield was detected at a higher initial glucose production, as this difference was indicated by the concentrations of glucose at the beginning of SSF, 17.66 g/l for commercial starch and 14.50 g/l for sago effluent hydrolysate. According to Zabed *et al.* (2014), initial sugar concentration is an important influencing parameter as it has a direct effect on the production and yield of ethanol.

The concentrations of fermentation co-products were also quantified by using the HPLC. Figure 3.7 illustrates that the concentration of lactic acid produced during SSF, with commercial starch showing overall slightly higher amounts than sago effluent hydrolysate. The production of lactic acid in batch culture with commercial starch surged in the first 6 hours (2.16 g/l) of SSF and peaked 12 hours (2.19 g/l). The concentration ranged from 2.11-2.19 g/l afterwards and dropped to 1.93 g/l at the end of fermentation. In sago effluent hydrolysate, lactic acid concentration reached its peak of 1.58 g/l at 24 h and decreased gradually to 1.22 g/l at 120 h. According to Graves *et al.* (2006), lactic acid will only inhibit ethanol production by *S. cerevisiae*, once its concentration is above 3.00% (w/v) or 30.00 g/l. Hence, in the current study the yeast metabolism and ethanol yield in both fermentation broths were not affected by lactic acid production, which were in the range of 1.58 g/l to 2.19 g/l (Oliva-Neto & Yokoya, 2001).

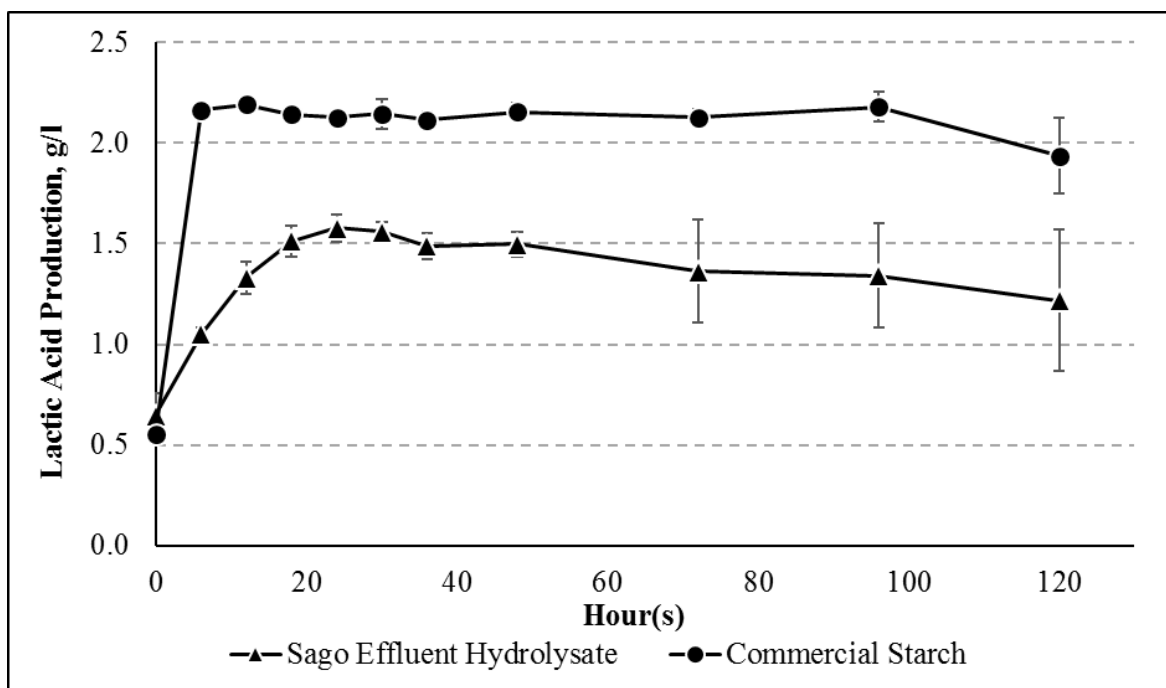


Figure 3.7: Time course of lactic acid production, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

Acetic acid, another co-product of lignocellulosic ethanol production process, showed a synergistic effect on ethanol yield with respect to glucose consumption (Casey *et al.*, 2010; Liu *et al.*, 2017; Oliva *et al.*, 2006). According to Erdei *et al.* (2010), ethanol yield is inhibited by high concentrations of acetic acid, specifically when greater than 10.00 g/l. As shown in Figure 3.8, the trend of acetic acid production in both sago effluent hydrolysate and commercial starch was relatively constant throughout the whole SSF process after the initial climb in the first 18 hours. At the end of fermentation, the concentration of acetic acid in sago effluent hydrolysate and commercial starch were 0.85 g/l and 0.91 g/l, respectively. Therefore, in the current study, there should be no inhibitory effect from acetic acid on the ethanol yield as only a small amount of acetic acid was produced throughout the SSF process.

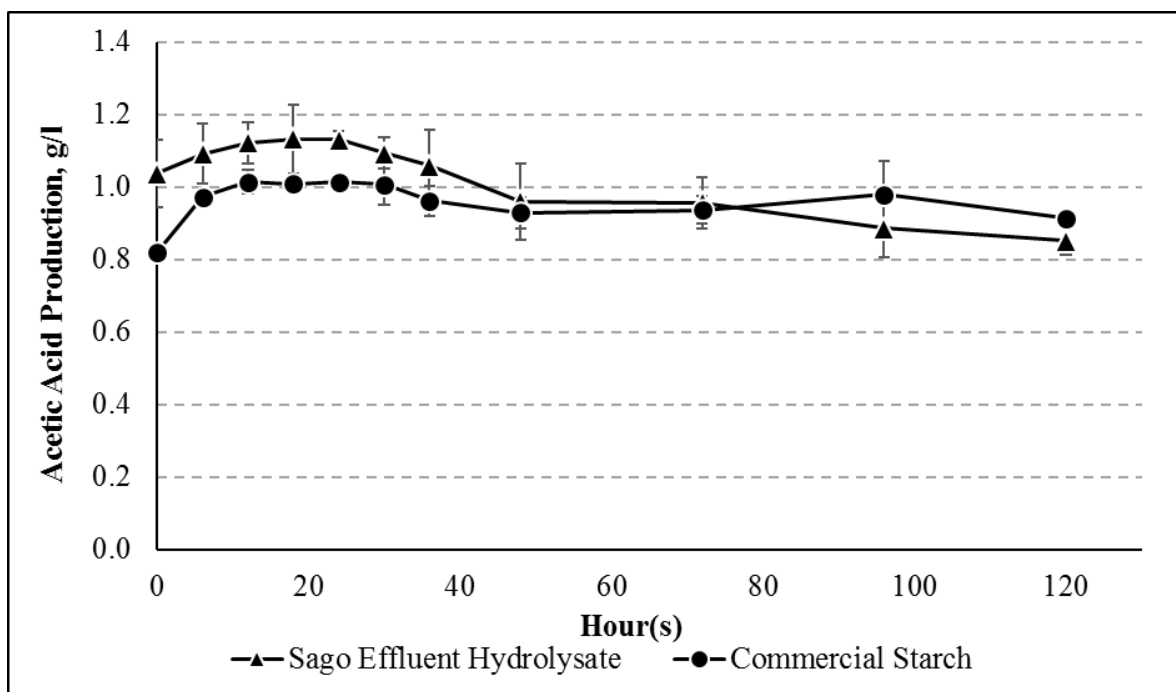


Figure 3.8: Time course of acetic acid production, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

Statistical analyses via One-Way Univariate ANOVA (F-Test) using IBM® SPSS® Statistics Software was performed and are summarised in Table 3.2. Studies validated the initial glucose concentration, lactic acid and acetic acid productions for both feedstocks were significantly different, with all p-values < 0.05. However, ethanol yield from sago effluent hydrolysate was statistical insignificantly compared to ethanol yield from commercial starch, with p-value > 0.05. Thus, the current study established that sago effluent hydrolysate has the same capacity with commercial starch when used as substrate by *S. cerevisiae* for bioethanol production. In a conclusion, sago starch in wastewater not only can be utilised as substrate for fermentative biohydrogen production (Hasyim *et al.*, 2011; Yunus *et al.*, 2014), but even has significant advantages when utilised for bioethanol production.

Table 3.2: Summary of univariate analysis of variance for both feedstocks.

Aspects	Sago Effluent Hydrolysate (g/l)	Comercial Starch (g/l)	R ²	p-value
Initial glucose	14.50 ± 0.62	17.66 ± 0.33	0.938	0.001
Lactic acid	1.33 ± 0.08	2.19 ± 0.02	0.988	0.000
Acetic acid	1.12 ± 0.06	1.01 ± 0.03	0.670	0.046
Ethanol yield	8.54 ± 0.44	8.88 ± 0.07	0.304	0.257

3.4 Conclusion

Ethanol production from carbohydrate-rich wastewater is one of the approaches to reduce both consumption of fossil fuels and environmental pollution. Industrial wastes like sago effluent have the potential to be the primary feedstock for bioethanol production. It is abundant and readily available, thus has a huge advantage over other sources. Industrial waste is the dominant material in landfills and its utilisation would alleviate land fill and other environmental issues. In this study, sago effluent hydrolysate was subjected to saccharification by amylolytic enzymes to obtain maximum fermentable sugars that were in turn being converted into ethanol by *S. cerevisiae*. Ethanol yield from sago effluent hydrolysate peaked at 12 h (8.39 ± 0.20 g/l or $84.89 \pm 4.56\%$ TEY) and was not significantly different compared to commercial starch according to F-test. Hence, compared to commercial starch, sago effluent hydrolysate was found to be a potentially excellent feedstock for bioethanol production via SSF, with many desirable characteristics.

CHAPTER 4

PRODUCTION OF BIOETHANOL FROM SAGO HAMPAS VIA SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

4.1 Introduction

Fuel demands from the transportation sector, which is the biggest user of diesel and gasoline, has increased tremendously in a worrying trend (Aditiya *et al.*, 2016). These fossil-based fuels are non-renewable (Vincent *et al.*, 2015a). They are also not environmentally, ecologically or economically sustainable in a long term (Adekunle *et al.*, 2016; Zabed *et al.*, 2014). Therefore, alternative liquid biofuels, such as bioethanol, is currently being mass produced to substitute and supplement petroleum-based fuel due to its carbon dioxide neutrality and sustainability (Adekunle *et al.*, 2016; Thangavelu *et al.*, 2016; Tye *et al.*, 2016; Vincent *et al.*, 2014). However, the current practices of using food and feed-based feedstock, such as corn and sugar cane, are also undesirable and controversial. Therefore, the utilisation of non-edible lignocellulosic materials for second-generation bioethanol production is capturing the attention of many researchers due to the restrictions in the use of food crops for first-generation bioethanol production as well as the high costs involved (Aditiya *et al.*, 2016; Daylan & Ciliz, 2016).

Non-edible lignocellulosic materials are essentially composed of cellulose, hemicellulose and lignin. These materials include waste streams from various sources such as food processing industries, forestry, agriculture, domestic and municipality origins. Additionally, starch is found in the composition of starchy-lignocellulosic biomass such as cassava pulp,

food waste and sago hampas (Behera *et al.*, 2014; Thangavelu *et al.*, 2016; Wan *et al.*, 2016). These resources do not entirely compete with agricultural crops, and are excellent candidates to be used as raw materials for bioethanol production (Tye *et al.*, 2016).

Sarawak (Malaysia) is the largest sago-growing area, and has been recognised as the biggest exporter of sago starch in the world. Sarawak exports approximately 44,000 tonnes of starch annually, mainly to Peninsular Malaysia, Japan and Singapore (Awg-Adeni *et al.*, 2012). However, the production of this precious commodity also generates wastes, in the forms of organic rich effluent and solid residues called sago hampas, that are discharged into nearby streams as well as deposited in the factory compound (Awg-Adeni *et al.*, 2010). These practices had led to several environmental issues such as water pollution and wastage of valuable resources (Wan *et al.*, 2016). The solid portion of this effluent is made of 58% starch. This property makes it relatively easier and less expensive to process compared to another lignocellulosic biomass (Vincent *et al.*, 2015b). More importantly, sago hampas can be found in abundance in a typical sago starch processing plant where an estimated 7 tonnes of sago pith waste are generated daily (Awg-Adeni *et al.*, 2010).

Therefore, in order to find a possible use for this misplaced solid residue, this study was performed to investigate bioethanol production from sago hampas at several loadings via simultaneous saccharification and fermentation (SSF) with the aid of commercial amylolytic and cellulolytic enzymes, in the presence of *Saccharomyces cerevisiae*. According to Vincent *et al.* (2015a), SSF is a method of choice for the production of second-generation bioethanol from lignocellulosic biomass. During SSF, resultant sugars liberated by the hydrolysing enzymes are simultaneously metabolised by fermenting microorganisms to

produce ethanol, thereby alleviating problems caused by product inhibition (Awg-Adeni *et al.*, 2012; Kannan *et al.*, 2013; Vincent *et al.*, 2015b). Other advantage of using SSF include lower enzyme dosage, higher ethanol yield, lower requirement for sterile conditions, shorter processing time and cost reduction by eliminating expensive reaction and separation equipment (Jessen *et al.*, 2012; Kang *et al.*, 2014; Triwahyuni *et al.*, 2015).

4.2 Materials and Methods

4.2.1 Characterisation of Sago Hampas

Sago hampas was collected from a local sago starch processing plant in Pusa, Sarawak. The sample moisture content was determined by weight loss from initial weight after oven-drying at 105 °C for 72 hours. Prior to lignocellulosic composition analysis, the sago hampas was destarched and dried for 3 days before it was ground to pass through a 1 mm (20 mesh) screen sieve. The samples were subjected to starch and fibre analysis using the phenol sulphuric acid assay, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF) and Klason Lignin Determination according to Goering and Van Soest (1970). Ash content was quantified by ashing samples at 500 °C (KC 40/13, ThermConcept, Germany) for 24 h. All analyses to characterize the sago hampas content were performed in triplicates (n=3).

4.2.2 *Saccharomyces cerevisiae* Culture Preparation

Saccharomyces cerevisiae ATCC 24859 culture inoculum for fermentation stage was prepared by growing the culture overnight in 150 ml of sterile Yeast Malt (YM) broth, in 250 ml conical flask with constant agitation at 120 rpm using a rotating shaker (NB-101MT

Multi Shaker, N-Biotek, Korea). The cell pellet was harvested through centrifugation (BK-1032J Low Speed Centrifuge, Biobase, China) in 50 ml falcon tube for 6 min at 3,240 x g. The supernatants were discarded and the cells were added into the fermentation broth during the initial stages of the simultaneous saccharification and fermentation (SSF).

4.2.3 Simultaneous Saccharification and Fermentation (SSF)

SSF were carried out in 500 ml Schott bottles with batch cultures of 200 ml volume consisting of yeast peptone citrate buffer (YP-CB, 50 mM) solution and sago hampas (2.5%, 5.0% and 7.5% w/v). The fermentation broth was autoclaved (Model No. 25X Electric Pressure Steam Steriliser, All American, United States) to provide sterility and to gelatinise the starch. Next, the broths were kept in a 50 °C oven for one hour prior to the addition of amylases (12.5 U/ml of α -amylase; 25.0 U/ml of glucoamylase) and cellulase (50 FPU/g cellulose). Once the temperature of the fermentation broth had cooled down to ambient temperature, harvested 24 hours old *S. cerevisiae* cells were inoculated aseptically. The fermentation was performed under anaerobic condition for five days with a constant agitation of 150 rpm. Sample aliquots of 1.5 ml samples were pipetted aseptically from each bottle at predetermined time intervals and centrifuged (*WiseSpin*® CF-10 High-Performance Microcentrifuge Set, Daihan Scientific, Korea) at 10,125 x g for 5 min. The supernatant was then filtered through 0.45 μ m nylon syringe filter (Whatman, NJ, USA) prior to phenol-sulphuric total carbohydrate assay and High Performance Liquid Chromatography (HPLC) analyses. At the end of fermentation period, the remaining biomass was centrifuged and dried for determination of biomass reduction.

4.2.4 Phenol-Sulphuric Total Carbohydrate Assay

The filtered supernatant was analysed for total carbohydrate via phenol-sulphuric acid (PSA) assay (Crawford and Pometto, 1988). Colour formation in the PSA was determined by measuring absorbance (SP-880 Visible Spectrophotometer, Metertech, Taiwan) against the reagent blank at 490 nm and the absorbance value was converted into equivalent sugar concentration based on a standard glucose curve constructed previously. All the analyses were performed in triplicate (n=3).

4.2.5 High Performance Liquid Chromatography (HPLC) Analysis

Filtered sample aliquots were also analysed using High Performance Liquid Chromatography (HPLC) for residual glucose, maltose/cellobiose, xylose and arabinose as well as fermentation products such as ethanol, acetic acid, lactic acid. The HPLC system (LC-20A, Shimadzu, Japan) used in this study was equipped with a column oven (CTO-20A, Shimadzu, Japan), refractive index detector (RID-10A, Shimadzu, Japan), isocratic pump (LC-20AT, Shimadzu, Japan) and computer controller. The separation and analysis of ethanol and other fermentation constituents were done on a Bio-Rad Aminex HPX-87H column (150 × 7.8 mm; Bio-Rad, CA, USA). Filtered aliquots of 20 µl were injected into HPLC using 5 mM H₂SO₄ as the mobile phase, operating at a rate flow of 0.8 ml/min with the column heated to 65 °C (Vincent *et al.*, 2015b).

4.2.6 Data Analysis

All experiments in this study were performed in triplicates and data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's *post hoc* tests for significant differences were performed for all final sets to determine multiple

comparisons of the production based on the feedstock loadings using IBM® SPSS® Statistics Software version 22 with a significance level of 0.05.

4.3 Results and Discussion

This study was conducted to utilise waste sago hampas to produce bioethanol via simultaneous saccharification and fermentation (SSF) at 2.5%, 5.0% and 7.5% (w/v) solid loadings to produce bioethanol. Prior to the SSF process, compositional analyses were done on the sago hampas fibre. The results, as presented in Table 4.1, show that the main components in dried sago hampas are starch ($55.40 \pm 0.02\%$), cellulose ($23.64 \pm 0.77\%$), hemicellulose ($9.07 \pm 1.18\%$), lignin ($4.01 \pm 0.51\%$) and ash ($2.23 \pm 0.01\%$). The high percentages of starch and cellulosic components are comparable to the previous studies by Awg-Adeni *et al.* (2012), Thangavelu *et al.* (2014) and Vincent *et al.* (2015b). These properties render sago hampas as a prime material for amylolytic and cellulolytic hydrolyses to be further used downstream for ethanolic fermentation (Arapoglou *et al.*, 2010).

Table 4.1: Compositional analysis of sago hampas (as percentage based on dry weight; n=3).

Chemical Composition	% w/w
Starch	55.40 ± 0.02
Cellulose	23.64 ± 0.77
Hemicellulose	9.07 ± 1.18
Lignin	4.01 ± 0.51
Ash	2.23 ± 0.01
Others	5.65 ± 0.09

Enzymatic saccharification is an important step prior to fermentation in yielding as much fermentable sugars as possible for the microbial conversion to ethanol. In this process, amylolytic enzymes, α -amylase and glucoamylase, were used as both α -1,4 and α -1,6-debranching hydrolases to break down gelatinised starch into maltose and glucose, for posterior saccharification and fermentation (Aydemir *et al.*, 2014). These amylolytic enzymes act synergistically during the successive enzymatic saccharification of starch in accordance to several previously reported studies (Diong *et al.*, 2016; Kannan *et al.*, 2013). Later, cellulolytic enzyme, consisting mainly of β -1,4-endoglucanase, β -1,4-exoglucanase and β -glucosidase, were added to disrupt the solid fibre structure at the solid-liquid interaction, making the individual fibre available for hydrolysis (Lakhundi *et al.*, 2015).

Total carbohydrate concentration in this study was determined via the phenol-sulphuric acid assay and the results are depicted in Figure 4.1. The initial total carbohydrates for 2.5%, 5.0% and 7.5% (w/v) sago hampas loadings were 18.26 g/l, 39.55 g/l and 56.53 g/l respectively; and the overall profile exhibited similar trends in spite of different feedstock loadings. Throughout the fermentation period, the total carbohydrates in batch system of 2.5% (w/v) feedstock loading decreased steeply at the first 6 h and then remained virtually constant. Meanwhile, the other two feedstock loadings decreased sharply at the first 12 h. Based on the results obtained, experiments with 7.5% (w/v) substrate showed the highest carbohydrates consumption of 87.0% (49.16 g/l) compared to the other substrate concentrations. However, residual carbohydrates were still detected in all fermentation media on the final day of SSF, indicating that not all resultant sugars were converted into ethanol.

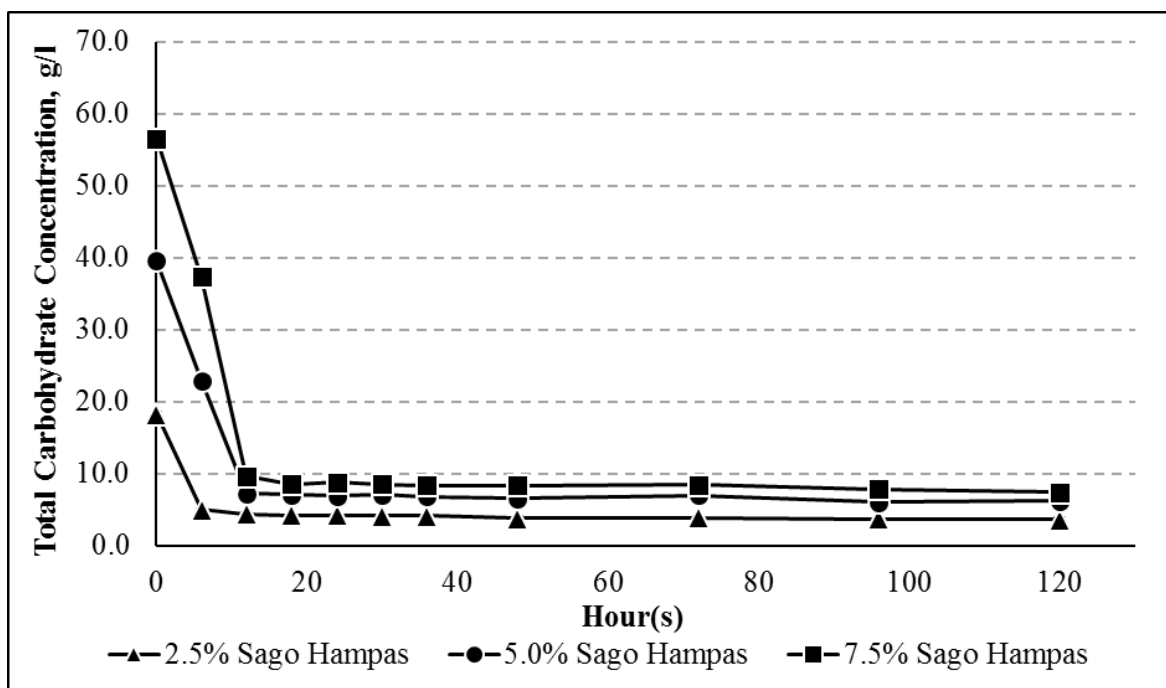


Figure 4.1: Time course of total carbohydrate concentration, as determined by the phenol-sulphuric acid assay. The data points represent the average of three independent experiments (n=3).

To supplement the carbohydrate assay, the efficiency of enzymes in hydrolysing the sago hampas were gauged and validated through HPLC analysis for the presence of the main monomeric end products from starch and cellulosic polymers (Vincent *et al.*, 2014). Figure 4.2 shows the glucose concentrations profiles were similar for the all feedstock loadings, as seen in the carbohydrate profiles. The initial glucose production in the broth with 5.0% (w/v) sago hampas loading showed a two-fold higher reading than 2.5% (w/v), which was 34.72 g/l compared to 18.29 g/l. However, at 0 h, the glucose concentration in the broth with 7.5% (w/v) was valued at 42.83 g/l only, due to the inefficient enzymatic saccharification of starch (Gupta *et al.*, 2012; Vincent *et al.*, 2015b). By 18 h, no glucose was detected in all samples; a good indication that efficient conversion of glucose into ethanol was achieved during fermentation.

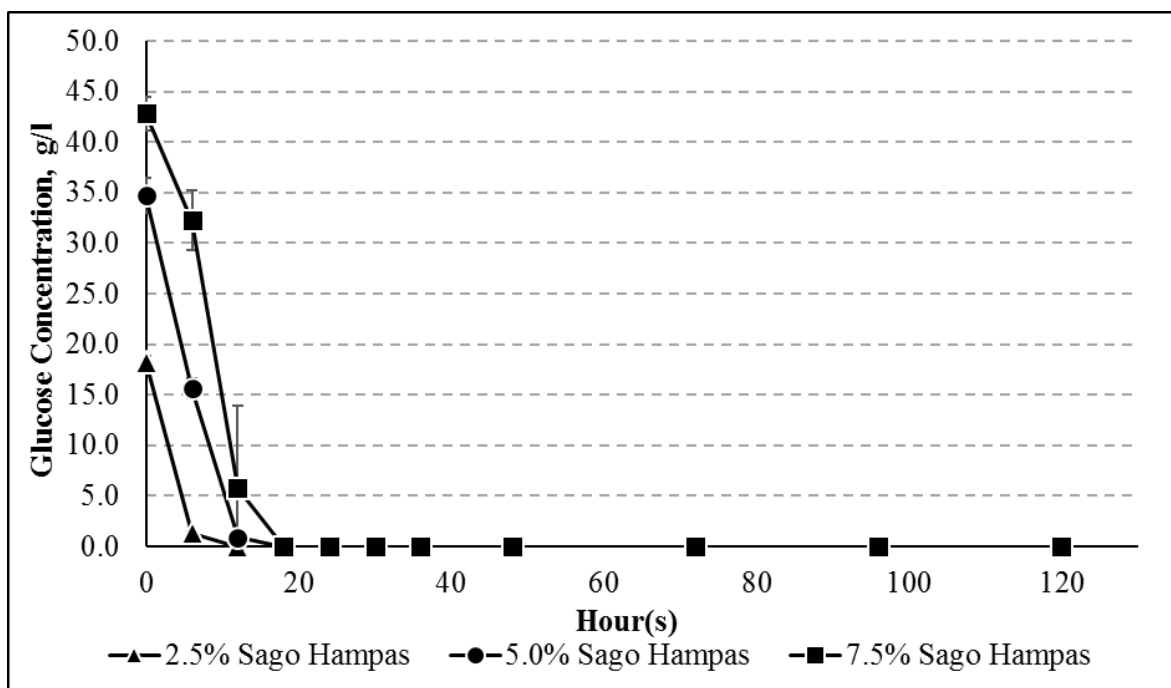


Figure 4.2: Time course of glucose concentration, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

Maltose/cellobiose are other saccharification products from starchy-cellulosic materials (Bhaumik & Dhepe, 2015; Su *et al.*, 2010). The initial maltose/cellobiose productions (Figure 4.3) were between 1.03 g/l to 1.54 g/l for all feedstock loadings. By 6 h, the early depletion of maltose/cellobiose was detected in fermentation with 2.5% (w/v) sago hampas loading. In contrast, maltose/cellobiose concentrations peaked at 2.22 g/l and 2.74 g/l for experiments with 5.0% (w/v) and 7.5% (w/v) sago hampas loads, respectively. The levels then decreased sharply until no maltose/cellobiose was further detected by 18 h, demonstrating the effectiveness of enzymatic conversion of maltose/cellobiose to glucose and promptly into ethanol by yeast (Ferreira *et al.*, 2010).

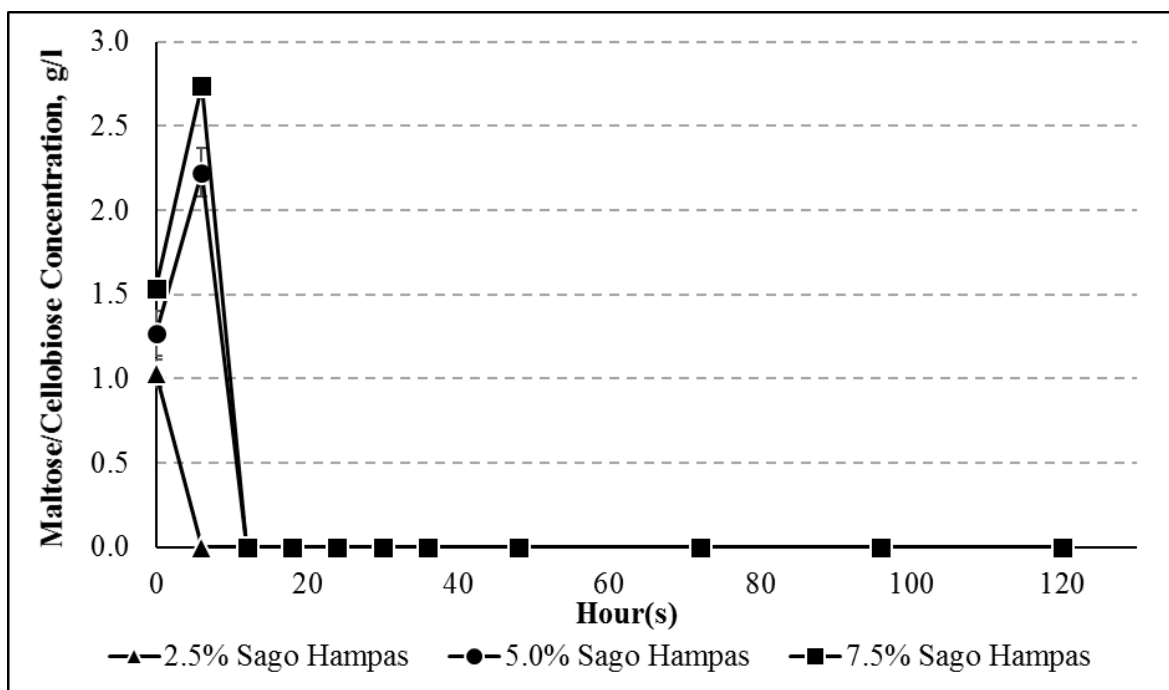


Figure 4.3: Time course of maltose/cellobiose concentration, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

Effective enzymatic hydrolysis of a typical lignocellulosic material, such as sago hampas, can be gauged not only by the glucose and maltose/cellobiose profile, but also by the amounts of xylose and arabinose released into the fermentation broth (Im *et al.*, 2016). This is anticipated when xylanases are part of the enzymes consortia used. The presence of xylanase is also highly vital as xylanase have synergistic effects on cellulose hydrolysis by degrading heterogeneous xylan polymer that surrounds cellulose microfibrils, resulting in an enhanced hydrolysis of the overall lignocellulosic biomass (Wi *et al.*, 2013). Figure 4 and Figure 5 show the xylose and arabinose production profiles in the study. In general, both productions increased throughout the SSF period. These observations could be explained by the inability of *S. cerevisiae* to efficiently utilise both sugars as sole carbon source and its conversion into ethanol (Cassa-Barbosa *et al.*, 2015; Srivastava *et al.*, 2015).

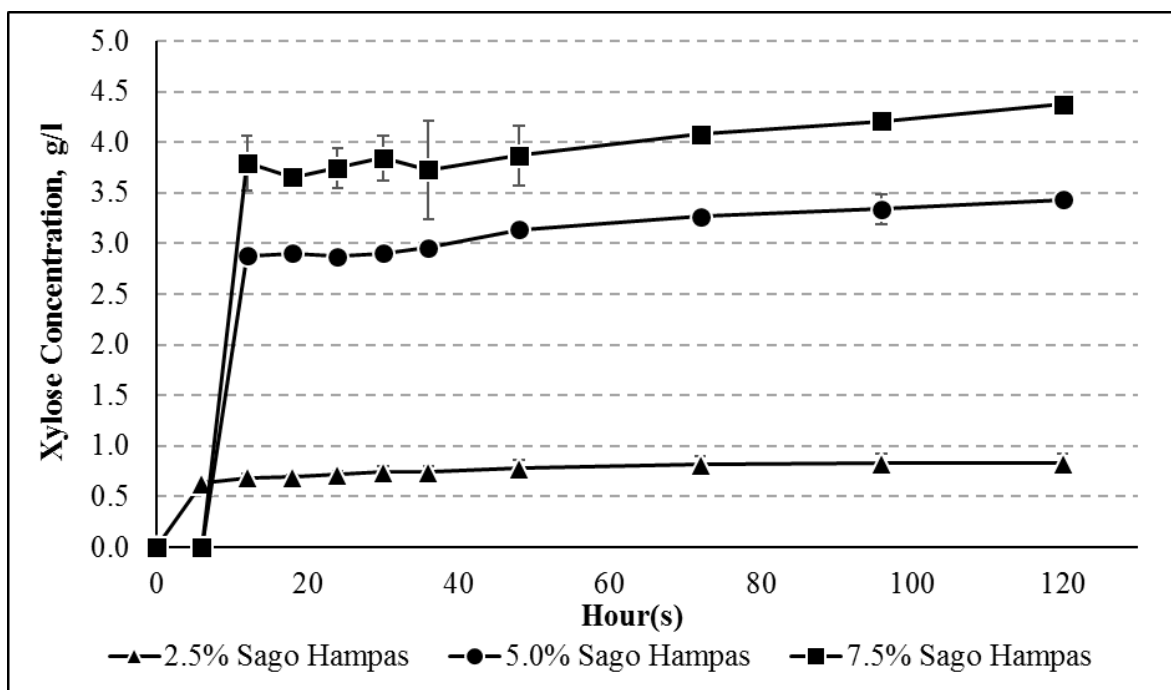


Figure 4.4: Time course of xylose concentration, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

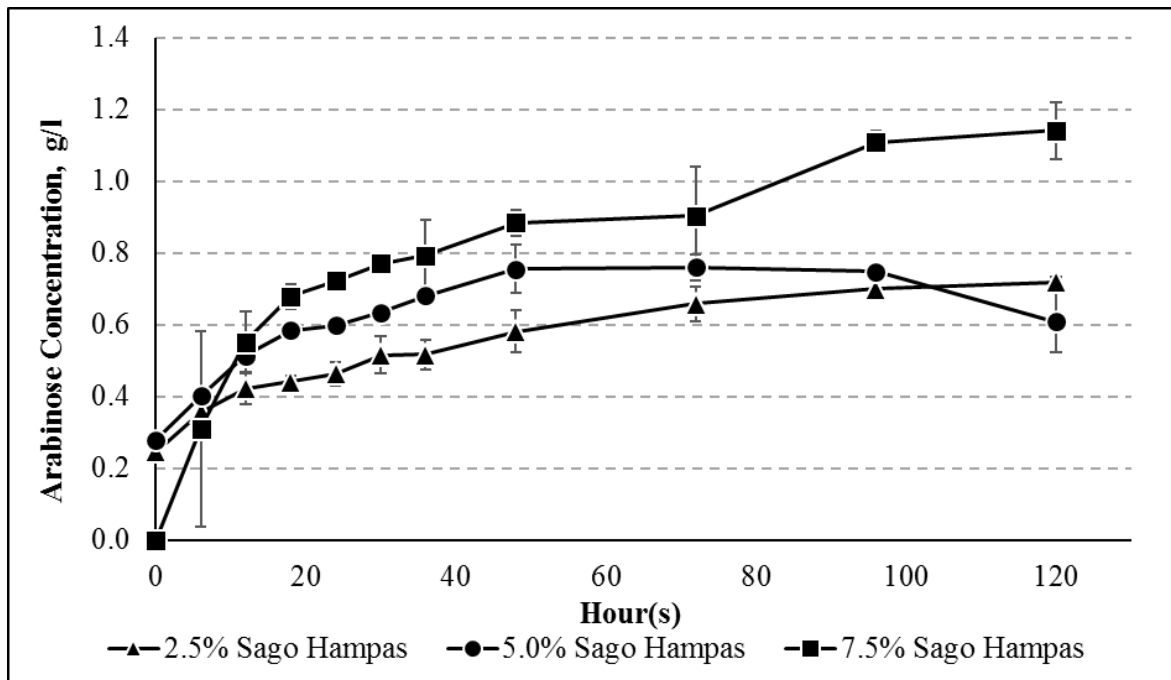


Figure 4.5: Time course of arabinose concentration, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

The highest theoretical ethanol yield (TEY) was recorded in broth contains 5.0% (w/v) sago hampas loading at 79.65% or 17.79 g/l at 48 h (Figure 4.6). This is significantly higher than TEY in the samples with 2.5% and 7.5% (w/v) sago hampas loadings, which were 75.0% TEY (8.38 g/l) and 69.48% TEY (23.38 g/l), respectively. For the experiment with 2.5% (w/v) sago hampas loading, ethanol production increased significantly to 75.00% TEY from 6 to 12 h, decreasing slightly thereafter until 120 h to 62.38% TEY. At 7.5% (w/v) sago hampas loading, theoretical yield of bioethanol production increased sharply to 61.77% TEY from 0 to 12 h, increasing slightly until reached its maximum ethanol production peaked at 48 h.

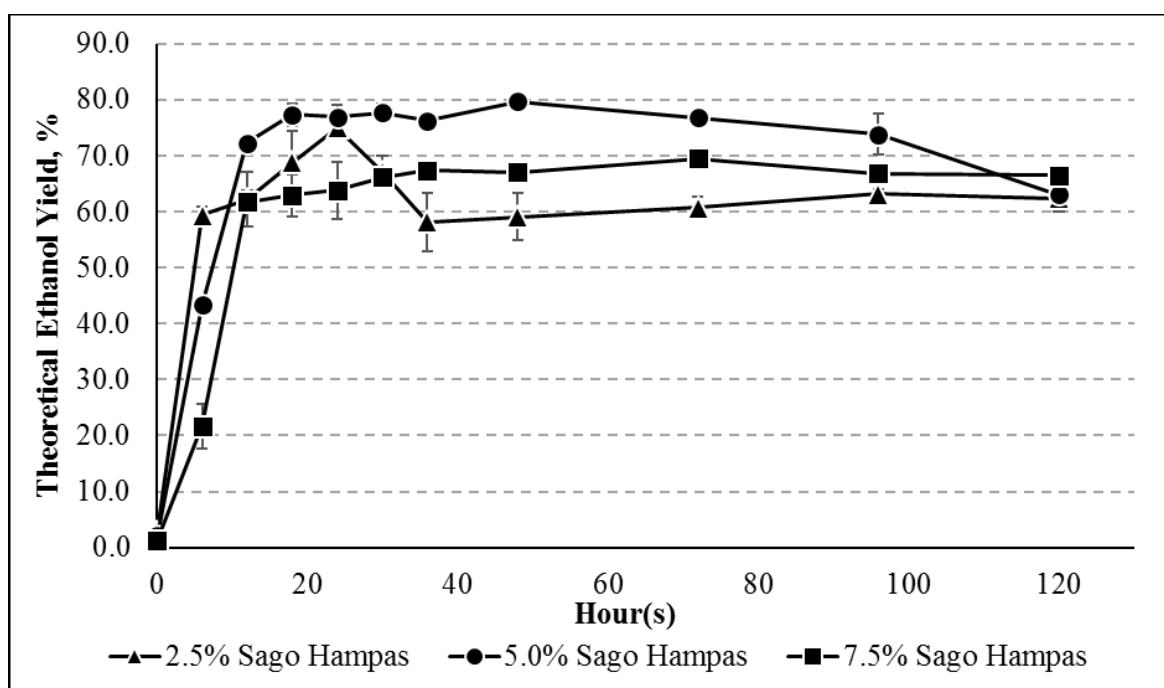


Figure 4.6: Time course of theoretical ethanol yield (TEY) profile during SSF process. [Note: The data points represent the average of three independent experiments (n=3).]

Based on the TEY profile (Figure 4.6), one notable observation was that these differences in the peak times for ethanol productions in all fermentation broths occurred inversely to the

fermentable sugars concentrations present in the fermentation media, as reported elsewhere (Vincent *et al.*, 2015a). The concentration of glucose (Figure 4.2) was higher at 7.5% sago hampas and more time was required for the *S. cerevisiae* to fully convert the glucose into ethanol, while, the broth with 2.5% (w/v) sago hampas had lower fermentable sugars concentrations, requiring a shorter period to fully convert the glucose into ethanol. Ethanol production profile for the experiment with 5.0% (w/v) sago hampas increased rapidly from 0 to 12 h (at 72.22% TEY) and remained at the range of 76.23% to 77.66% TEY. At 48 h, ethanol production increased slightly to 79.65% TEY but decreased to 63.06% TEY at 120 h. Further statistical analysis, as showed in Figure 4.7, validated the statistically significant ethanol yield among the different feedstock loadings, with a p-value < 0.05. As concluded, the most effective yield of bioethanol was from sago hampas at 5.0% (w/v) loading.

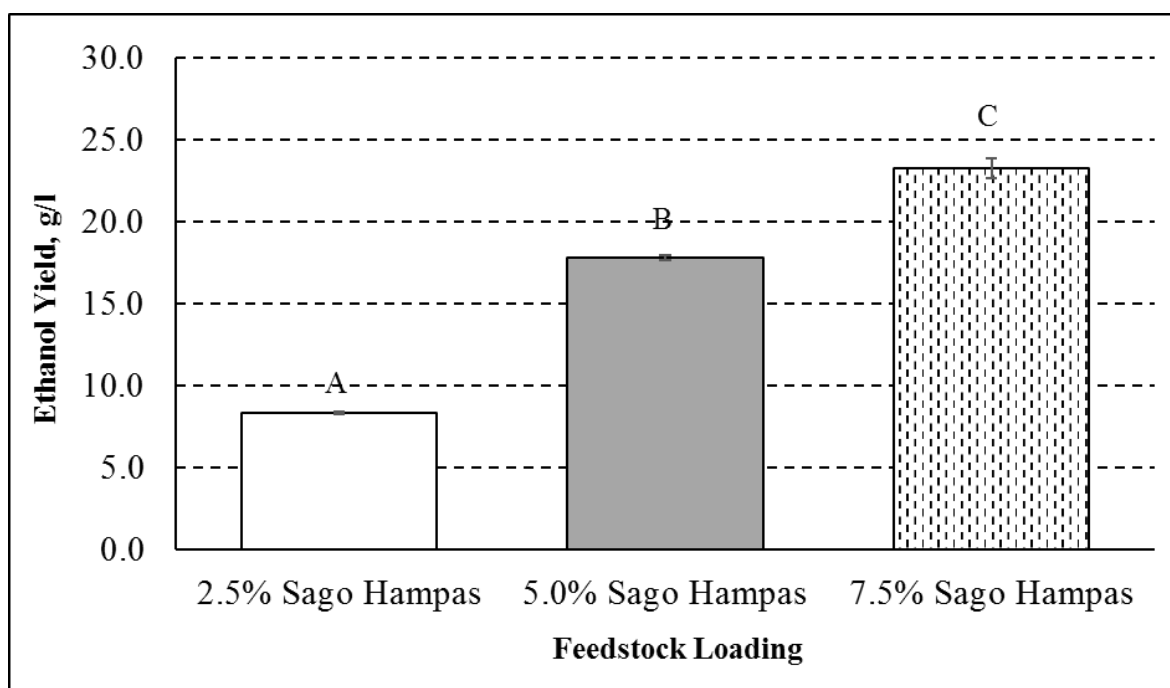


Figure 4.7: Maximum ethanol yield using different feedstock loadings. [Note: Letters on top of column validate statistically significant differences via Tukey's *post hoc* test, $\alpha=0.05$.]

It is essential to compare the ethanol yield from sago hampas reported previously. Thangavelu and his co-workers (2014) obtained only maximum 40.50% TEY from sago pith waste using microwave hydrothermal hydrolysis accelerated by carbon dioxide. Kannan *et al.* (2013) yielded 60.20% TEY from sago hampas treated with sulfuric acid supported by microwave irradiation. Additionally, based on previous studies by Vincent *et al.* (2015a; 2015b), sequential saccharification and simultaneous fermentation (SSSF) and simultaneous co-saccharification and fermentation (Sc-SF) with 5.0% (w/v) pretreated physically of sago hampas exhibited highest bioethanol productions at 77.43% TEY and 80.50% TEY respectively (Vincent *et al.*, 2015a; Vincent *et al.*, 2015b). Hence, the ethanol yield at 5.0% (w/v) sago hampas loading in the present study was favourably comparable to those described in the previous works (Kannan *et al.*, 2013; Thangavelu *et al.*, 2014; Vincent *et al.*, 2015a; Vincent *et al.*, 2015b).

Throughout the SSF, fermentation by-products such as lactic acid and acetic acid were also measured using HPLC. Based on Figure 4.8, lactic acid was produced at approximately 0.80 g/l for all experiments to their peaks at different times. The highest lactic acid concentration was recorded in the broth with 7.5% (w/v) sago hampas at 3.69 g/l compared with 2.5% (w/v) at 1.86 g/l and 5.0% (w/v) at 2.98 g/l. Figure 4.9 shows the acetic acid production profiles. At 24 h, acetic acid production in broth with 2.5% (w/v) sago hampas peaked at 1.49 g/l, and, remained virtually constant until the end of fermentation. Meanwhile, in the broth with 5.0% and 7.5% (w/v) sago hampas, acetic acid increased steadily until 120 h to final concentrations of 1.85 g/l and 2.07 g/l, respectively. One possible explanation for the increment in the rate of acetic acid production is the biological oxidation of ethanol. Additionally, the presence of acetic acid is also an important indicator to evaluate the

efficiency of biomass hydrolysis, as the increase in acetic acid levels indicates continuous degradation of lignocellulosic materials during SSF (Vincent *et al.*, 2015a).

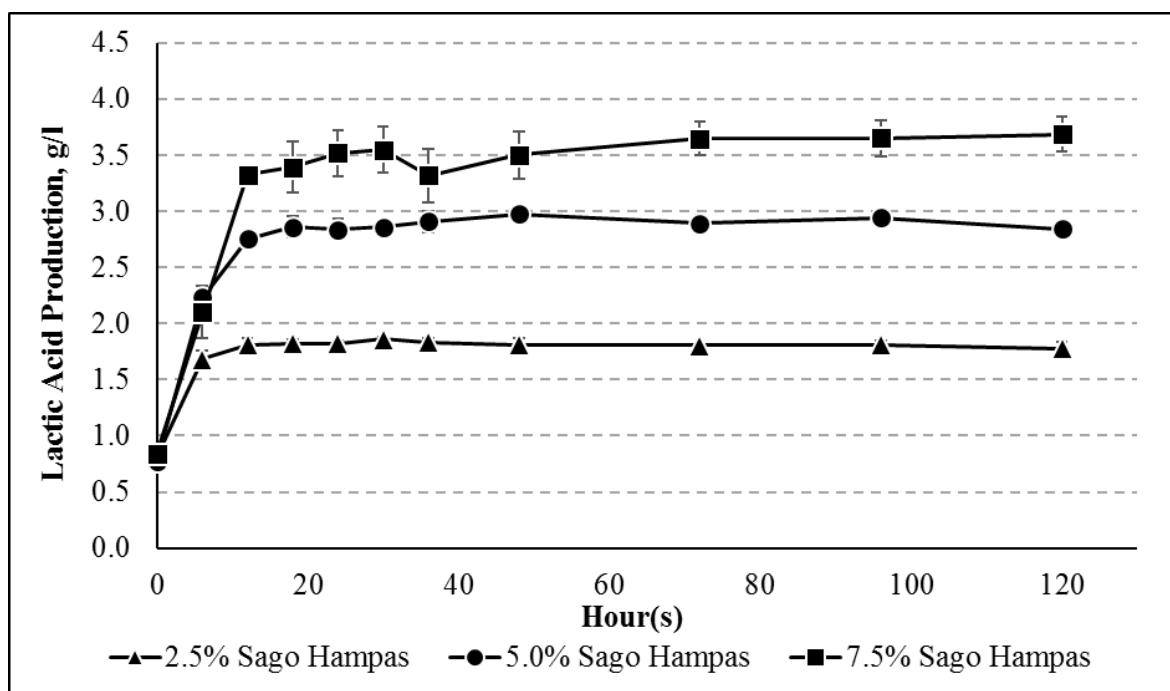


Figure 4.8: Time course of lactic acid production, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

By the end of the SSF period, total biomass reduction was recorded between 66.27% to 71.33% (Figure 4.10), indicating that less than 35.00% of the original substrate remained unutilised. These observations are encouraging as this study have demonstrated that bioethanol production from fresh sago hampas may assist in relieving the environmental burden from the sago waste released by starch producing industries. Eventually, this may alleviate the needs for alternative waste management (Kannan *et al.*, 2013; Linggang *et al.*, 2012).

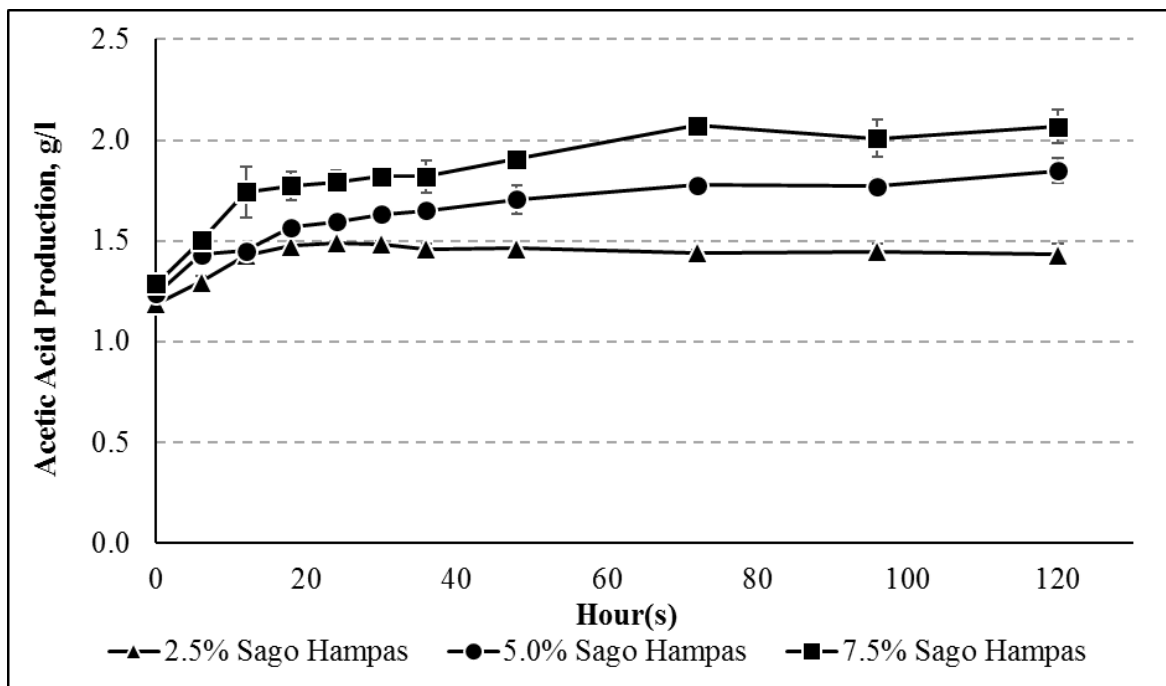


Figure 4.9: Time course of acetic acid production, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

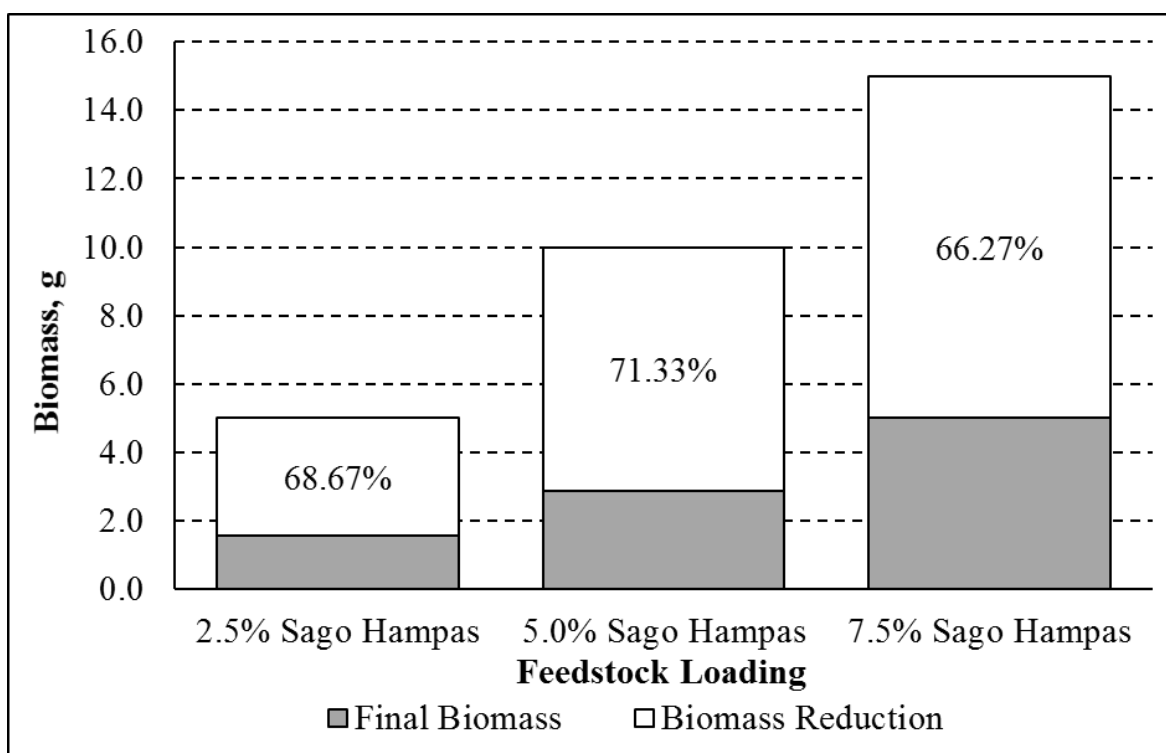


Figure 4.10: Total biomass reduction by the end of the SSF process. [Note: The data points represent the average of three independent experiments (n=3).]

4.4 Conclusion

From this study, fresh sago hampas is shown to be potential bioethanol feedstock when subjected to simultaneous saccharification and fermentation (SSF) with the aid of amylolytic-cellulolytic enzymes and *Saccharomyces cerevisiae*. The results showed that SSF with 5.0% (w/v) sago hampas loading was the most effective in generating higher ethanol yield at 79.65% of TEY in comparison to SSF with 2.5% and 7.5% (w/v) sago hampas produced ethanol at only 8.38 g/l (75.00% TEY) and 23.28 g/l (69.48% TEY).

CHAPTER 5

UTILISATION OF TOTAL SAGO EFFLUENT AS FEEDSTOCK FOR BIOETHANOL PRODUCTION VIA SEQUENTIAL SACCHARIFICATION AND SIMULTANEOUS FERMENTATION (SSSF)

5.1 Introduction

Bioethanol, an important liquid biofuel, has been advocated as the most promising renewable fuel to tackle the problems related to rising crude oil prices, global warming and the inevitable depletion of petroleum reserves (Saini & Saini, 2015). Bioethanol is usually produced by microbe-catalysed fermentations using plant biomass as raw material via two platforms, the first- and second-generation bioethanol productions (Pietrzak & Kawa-Rygielska, 2014; Vincent *et al.*, 2015b). Starchy materials and sugar-based crops are predominant first-generation bioethanol feedstocks at the industrial level (Saifuddin & Hussain, 2011). However, the disposal of cellulose and hemicellulose during the production is economically unfavourable as these constitute most of the carbon resource of the plants (Ritslaid *et al.*, 2010). In addition, the use of food crops as bioethanol feedstocks is competing with the allocation intended for human consumption (Welker *et al.*, 2015). Nevertheless, recent research efforts have extended to new alternative resources that are renewable, abundant and relatively inexpensive, while producing a reasonable yield and not competing with human consumption or other uses (Comwien *et al.*, 2015).

The inedible parts of plants, known as the lignocellulose biomass, are generated in large quantities through agricultural and forestry activities as well as residual waste from fruit and

vegetable processing operations (Chandel *et al.*, 2010). Much of the lignocellulosic biomass is often disposed of by burning, resulting in air pollution and emission of greenhouse gases (Singh *et al.*, 2014). Hence, utilisation of lignocellulosic biomass has gained increasing research interests particularly due to its structural carbohydrate such as cellulose and hemicellulose, which can potentially be converted into lignocellulosic-based bioethanol (Anwar *et al.*, 2014; da Silva *et al.*, 2016).

In Malaysia, the growing demands for sago starch have concomitantly increased the volume of sago effluent discharged from sago mills (Sangeetha *et al.*, 2015). Sago palm or *Metroxylon sagu* (Rottboll) belongs to a group of starch-bearing palms found in the Malay Archipelago and many tropical countries (Jalil *et al.*, 2015; Yahya *et al.*, 2011). This particular palm has been identified as a highly efficient carbohydrate and energy crops with the ability to accumulate high carbohydrate yields of up to 84.7% in its trunk (Abbas *et al.*, 2010; Abbasa & Ehara, 2012; Jalil *et al.*, 2015). However, the effluent has an obnoxious odour, irritating colour, low pH, high chemical oxygen demand (COD) and biological oxygen demand (BOD) content (Ruban *et al.*, 2013). It is usually disposed of into nearby waterways with copious fibrous residues from the extraction of starch from the sago trunk (Yunus *et al.*, 2014). This results in serious contamination to the environment (Hasyim *et al.*, 2011; Vincent *et al.*, 2015a; Wan *et al.*, 2016). Therefore, vigorous research efforts have been proposed to solve the current predicament faced by the Malaysian sago industry due to improper management of these wastes (Yunus *et al.*, 2014).

Total sago effluent (TSE) is a carbohydrate-rich wastewater that comprised of 94.0%-97.00% sago liquid residue while the remaining is sago solid waste or sago hampas (Awg-

Adeni *et al.*, 2010). According to previous studies, sago solid waste is convertible into bioethanol via hydrolysis and fermentation, but none has currently documented the utilisation of TSE as bioethanol feedstock (Awg-Adeni *et al.*, 2012; Kannan *et al.*, 2013; Thangavelu *et al.*, 2014; Vincent *et al.*, 2015a, 2015b). According to Comwien *et al.* (2015), carbohydrate-rich wastewater may contain relatively high and diverse levels of monosaccharides and disaccharides (glucose, mannose, xylose and arabinose), as well as more complex polymers (starch, cellulose and hemicellulose). Hence, sago effluent makes a promising feedstock for bioethanol production while simultaneously reduce environmental issues and wastage of valuable energy (Hasyim *et al.*, 2011; Wan *et al.*, 2016).

Even after the employment of efficient procedures during sago starch extraction, sago effluent still contains approximately 2.0%-3.00% residual starch with a similar percentage of fibre, making it an ideal feedstock for bioethanol production (Bujang, 2014). Starchy material like sago effluent is well known as a high yield feedstock, but its hydrolysis products are strongly inhibitory to amylolytic enzymes (Ajibola *et al.*, 2012; Bialas *et al.*, 2014). This bottleneck can be overcome by performing simultaneous saccharification and fermentation (SSF), where enzymatic saccharification and fermentation occur simultaneously in same vessel (Bialas *et al.*, 2014). However, the carbohydrate-rich TSE is also unfriendly towards the efficiency of cellulolytic enzymes used for the purpose of cellulose degradation. Hence, in this study, production of ethanol was carried out via a sequential saccharification and simultaneous fermentation (SSSF) process, the modified version of SSF involving amylolytic enzymes, cellulolytic enzyme and *Saccharomyces cerevisia* (Vincent *et al.*, 2015a).

5.2 Materials and Methods

5.2.1 Experimental Design

An overview of the bioethanol production using total sago effluent (TSE) is shown in Figure

5.1. All experiments including analytical methods were conducted in triplicates (n=3).

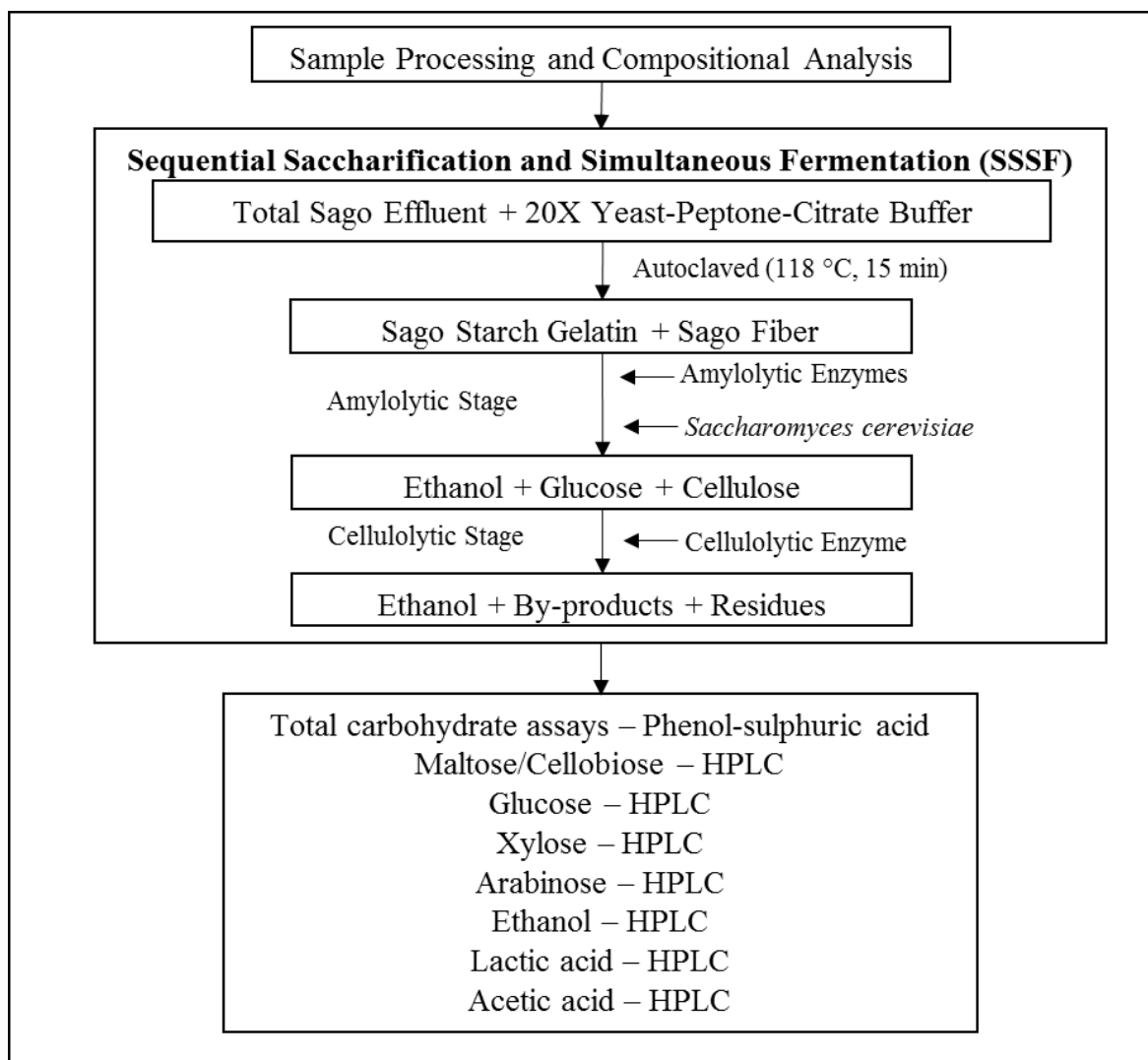


Figure 5.1: Experimental setup for bioethanol production using total sago effluent via SSSF

5.2.2 Sample Processing and Compositional Analysis

Fresh total sago effluent (TSE) was obtained from the Herdsen Sago Mill in Pusa, Sarawak. The TSE was centrifuged and oven-dried to determine the dry matter content. For compositional analysis, the sample was subjected to starch content and fibre analysis using phenol-sulphuric total carbohydrate assay, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF), and Klason Lignin Determination according to Goering and Van Soest (1970). Ash content was measured using standard protocols. All analyses to characterise the total sago effluent were conducted in triplicates (n=3).

5.2.3 *Saccharomyces cerevisiae* Culture Preparation

Saccharomyces cerevisiae (ATCC 24859) inoculum for the fermentation stage was prepared by growing the culture in 150 ml sterile Yeast Malt Broth (YMB) in 250 ml conical flasks with constant agitation at 120 rpm (NB-101MT Multi Shaker, N-Biotek, Korea) overnight, at ambient temperature. The culture was transferred into three 50 ml falcon tubes in equal volumes and centrifuged (BK-1032J Low Speed Centrifuge, Biobase, China) at 3,240 x g for 6 min to harvest the yeast cells (Vincent *et al.*, 2011). The supernatants were discarded and the cells were used as inoculums in the batch culture medium for the first stage of the sequential saccharification and simultaneous fermentation (SSSF).

5.2.4 Sequential Saccharification and Simultaneous Fermentation (SSSF)

SSSF was carried out in two stages: stage one was the amylolytic step, followed by the cellulolytic step in stage two. SSSF was conducted in 500 ml Schott bottles with batch culture of 200 ml consisting of 1X Yeast-Peptide-Citrate Buffer (YP-CB 0.05 M) and TSE. Next, the batch culture was autoclaved (Model No. 25X Electric Pressure Steam Steriliser,

All American, United State) for sterilisation and starch gelatinisation. During the amylolytic step, amylases (12.5 U/ml of α -amylase and 25.0 U/ml of glucoamylase; Sumson, China) were added after the batch cultures cooled to 60 °C and were left to further cool down to 37 °C before inoculating with *S. cerevisiae*. The batch cultures were then constantly agitated at 150 rpm under anaerobic condition for 24 hours at ambient temperature to allow the SSSF to complete the first stage. At 24 h, most of the gelatinised starch were liquefied and converted into glucose and ethanol, with the remaining solids mainly cellulosic fibres. The cellulolytic stage was then initiated by adding cellulase (50 FPU/g fibre; Sumson, China) and the batch culture was maintained until the fifth day to allow further digestion of the cellulosic components to produce more ethanol.

5.2.5 Sample Collection

Aliquots of 1.50 ml of samples were taken aseptically at predetermined time intervals (0, 6, 12, 18, 24, 30, 36, 48, 72, 96 and 120 h) and centrifuged (*WiseSpin* CF-10 High-Performance Microcentrifuge Set, Daihan Scientific, Korea) at 10,125 x g for 5 min. The supernatant was then filtered through a 0.45 µm sterile nylon syringe filter (Whatman, NJ, USA) prior to analysis. All experiments and analytical methods were conducted in triplicates. The results obtained were mean values with standard deviations (n=3).

5.2.6 Phenol-Sulphuric Total Carbohydrate Assay

Total carbohydrate content of the filtered supernatant was measured via phenol-sulphuric acid assay (Crawford & Pometto, 1988) at 490 nm (SP-880 Visible Spectrophotometer, Metertech, Taiwan). The equivalent sugar concentration (g/l) was determined based on a standard glucose concentration curve, which was generated prior to the assay.

5.2.7 High Performance Liquid Chromatography (HPLC) Analysis

The filtered sample in aliquots of 20 µl were analysed for residual sugars and fermentation products such as ethanol, lactic acid and acetic acid by injecting into the High Performance Liquid Chromatography (LC-20A, Shimadzu, Japan) equipped with a Prominence column oven (CTO-20A, Shimadzu, Japan), refractive index detector (RID-10A, Shimadzu, Japan), isocratic pump (LC20AT, Shimadzu, Japan) and computer controller. The separation and analysis of ethanol and other fermentation constituents were performed on a 150 x 7.8 mm Aminex HPX-87H column (Bio-Rad, CA, USA) with 0.005 M H₂SO₄ as the mobile phase, at a rate flow of 0.8 ml/min and a column temperature of 65 °C. The theoretical ethanol yield (TEY) was calculated according to the formula below:

$$\text{TEY (\%)} = \frac{\text{Ethanol produced (g/l)}}{\text{Theoretical ethanol produced (g/l)}} \times 100\%$$

5.3 Results and Discussion

The compositional analysis revealed that the bulk of total sago effluent (TSE) comprises of 97.00 ± 0.02% sago effluent hydrolysate and 3.00 ± 0.02% sago hampas. The result obtained was comparable to the data reported previously (Awg-Adeni *et al.*, 2012). The main components of sago effluent hydrolysate and sago hampas were starch, cellulose, hemicellulose, lignin and ash (Table 5.1). Based on the analysis, sago effluent hydrolysate contained 2.00 ± 0.02% starch; sago hampas contained 55.40 ± 0.02% starch, 23.64 ± 0.77% cellulose, 9.07 ± 1.18% hemicellulose, 4.01 ± 0.51% lignin and 2.23 ± 0.01% ash in dry

basis (w/w). Preliminary results suggested that TSE is ideal as bioethanol production feedstock.

Table 5.1: Compositional analysis of total sago effluent.

Compositions		%
Sago effluent hydrolysate	Water	95.00 $\pm$ 0.02
	Total Carbohydrate	2.00 $\pm$ 0.02
Total Sago Effluent	Starch	1.66 $\pm$ 0.00
	Cellulose	0.71 $\pm$ 0.02
	Hemicellulose	0.37 $\pm$ 0.04
	Lignin	0.12 $\pm$ 0.02
	Ash	0.07 $\pm$ 0.00
	Others	0.17 $\pm$ 0.00

Effective fermentation process is very critical to ethanol production yield (Bialas *et al.*, 2014). Simultaneous saccharification and fermentation (SSF) is a method of choice for bioethanol production from lignocellulosic biomass as well as from starchy materials (Bialas *et al.*, 2014; Vincent *et al.*, 2015a). It is a process that combines the enzymatic saccharification of complex polymers and fermentation of resultant sugars into ethanol in the same vessel at the same time (Ask *et al.*, 2012; Vincent *et al.*, 2015a). This process is shown to be superior to separate hydrolysis and fermentation (SHF) by minimising the inhibition of accumulated sugars on ethanol yield, thus reducing the potential loss of resultant sugars and is cost effective (Ask *et al.*, 2012; Ruiz *et al.*, 2012; Triwahyundi *et al.*, 2015).

However, the bottleneck in the SSF process is the high percentage of hydrolysis products, mainly glucose, which is inhibitory to the activities of enzymes (Bialas *et al.*, 2014). Hence, in this study, the SSF process was modified into a sequential process termed as sequential

saccharification and simultaneous fermentation (SSSF). According to Vincent *et al.* (2015a), this method can be implemented to further reduce the organic load in carbohydrate-rich feedstocks and ultimately produce more bioethanol. Therefore, the study was extended to a two-step process of enzymatic saccharification, which are the amylolytic step and the cellulolytic step on the sago effluent, in the presence of *Saccharomyces cerevisiae* as the fermenting microorganism. α -Amylase (12.5 U/ml) and glucoamylase (25.0 U/ml) were added during the amylolytic stage to stimulate the breakdown of the starch into glucose, by cleaving the 1,4- α -D-glycosidic linkages to liberate glucose as the end product (Singh *et al.*, 2016; Slivinski *et al.*, 2011). During the cellulolytic stage, cellulase (50 FPU/g cellulose) was added to further degrade cellulosic components in the sago hampas into glucose by breaking the 1,4- β -D-glycosidic linkages (Vincent *et al.*, 2015a). SSSF was conducted for five days according to a previous study that suggested a longer fermentation period provides the necessary information on the completion of ethanol production (Vincent, 2010).

During the 5-day SSSF process, the concentration of residual sugars and fermentation products were measured. As shown in Figure 5.2, a high total carbohydrate concentration of 36.72 g/l was recorded at 0 h, indicating the carbohydrate-rich nature of the TSE. The total carbohydrate concentration was decreased steeply to 1.97 g/l at 12 h, followed by a slight increase to 3.56 g/l at 30 h, marking the starting of the cellulolytic stage. The total carbohydrate remained in the range of 3.50 g/l to 4.03 g/l until the end of the fermentation period. On the other hand, the main monomeric sugar, glucose, was produced immediately at 0 h (33.86 g/l), signalling the initial saccharification of starch by the amylases (Vincent *et al.*, 2015b). By 24 h, glucose depletion was recorded during the cellulolytic stage due to its complete conversion by the yeast cells.

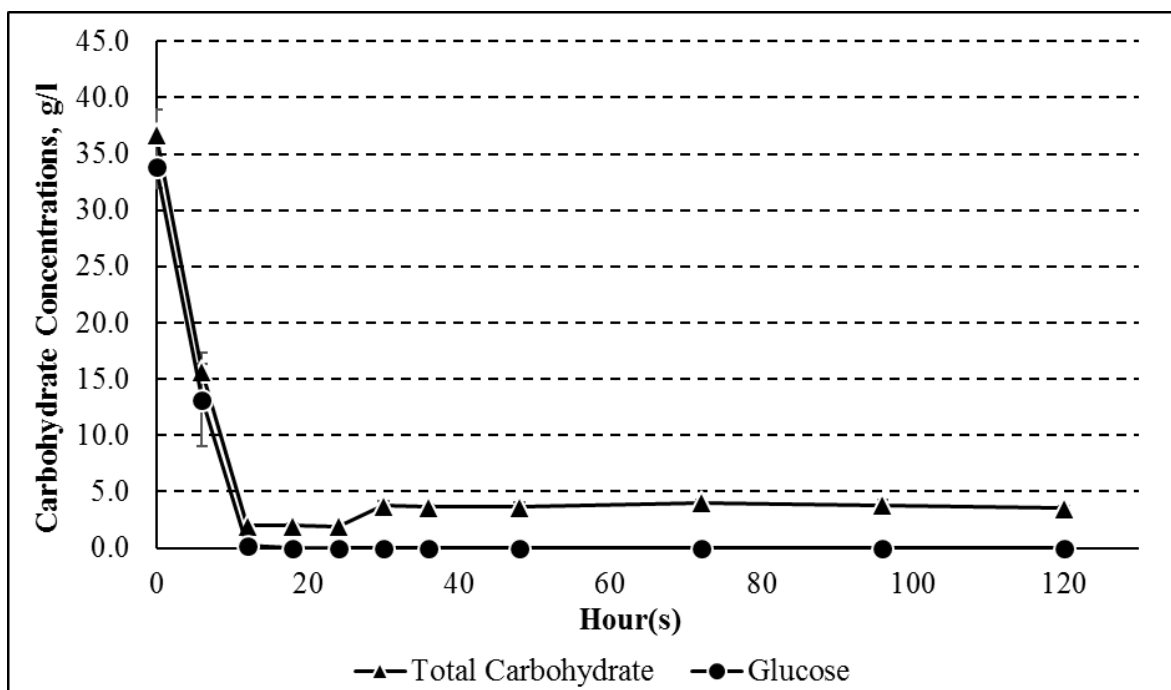


Figure 5.2: Time course of total carbohydrate concentration, as determined via the phenol-sulphuric method and time course of glucose concentration, as determined by HPLC. The data points represent the average of three independent experiments (n=3).

Maltose and cellobiose, products of SSSF from the starchy-lignocellulosic feedstock, were detected at 0 h with a value of 1.01 g/l (Figure 5.3), suggesting the partial enzymatic hydrolysis of starch and cellulose into disaccharides. However, there were no maltose or cellobiose observed from 24 h onwards, as they were being hydrolysed into glucose and ultimately ethanol. The synergetic enzymatic hydrolysis by glucoamylase and cellulase supplemented with β -glucosidase greatly reduced the inhibition caused by accumulation of sugars, thereby effectively improved the saccharification and fermentation (Bialas *et al.*, 2014; Slivinski *et al.*, 2011; Quiroz-Castañeda & Folch-Mallol, 2013).

Throughout the SSSF process, aldopentoses such as xylose and arabinose, which is derived from hemicellulose, were detected due to the addition of xylanase as ancillary enzyme. A low concentration of arabinose was produced immediately during the amyolytic stage at

0.23 g/l. According to Awg-Adeni *et al.* (2012), plant cell wall consists of microstructural cellulose embedded in a matrix of polysaccharides and proteins, surrounded by an outer layer of pectin material. A possible explanation for this observation was that autoclaving led to the degradation of the pectin backbone through β -elimination, producing arabinose at the beginning of the SSSF (Ralet *et al.*, 2009). Figure 5.3 shows that the production of xylose and arabinose were produced constantly during the cellulolytic stage. At the end of the SSSF period, a low concentration of xylose and arabinose were recorded, 0.84 g/l and 0.73 g/l, respectively. The accumulation of both sugars could be correlated to the hydrolysis of hemicellulose by xylanase and the inability of *S. cerevisiae* to utilise pentoses.



Figure 5.3: Time course of fermentable sugars such as maltose/cellobiose, xylose and arabinose concentrations, as determined by HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

Figure 5.4 shows the concentration profiles of the total carbohydrate, glucose, ethanol and the corresponding theoretical ethanol yield. Glucose that resulted from enzymatic hydrolysis

was rapidly consumed by *S. cerevisiae*, as indicated by the decrease in concentration from 0 to 12 h. Meanwhile, the concentration of ethanol increased rapidly from 0.69 g/l at 0 h to 12.69 g/l at 12 h, which is expected from the stoichiometry of the conversion of glucose into ethanol by fermenting microorganism in alcoholic fermentation process. During the cellulolytic stage, the production of ethanol increased slightly, due to residual starch and the low amount of resultant fermentable sugars from the breakdown of cellulose by cellulase. The SSSF process was completed after 48 h as the highest ethanol concentration from the sago effluent of 20.01 g/l was recorded, corresponding to a Theoretical Ethanol Yield (TEY) of 86.54%. It is noted that the latency to maximum ethanol production coincided with glucose depletion. Eventually the concentration of ethanol decreased, indicating cessation of the fermentation process.

Vincent *et al.* (2015a) reported that ethanol yield was at 14.13 g/l (77.43% of TEY) using a similar SSSF process with 5.0% (w/v) sago hampas as raw material. As a fermentation medium, the fermentative biohydrogen production from starch-containing synthetic wastewater as well as sago effluent have been reported (Hasyim *et al.*, 2011; Yunus *et al.*, 2014). Apart from that, wastewaters from the soft drink industry were examined as bioethanol media using yeast-mediated fermentation (Isla *et al.*, 2013). Another industrial waste, desizing wastewater from dye-bleaching industry has also been documented to be a possible economical medium for bioethanol production by direct fermentation of starch into ethanol using a co-culture system with *Bacillus subtilis* and *S. cerevisiae* (Tantipaibulvut *et al.*, 2015).

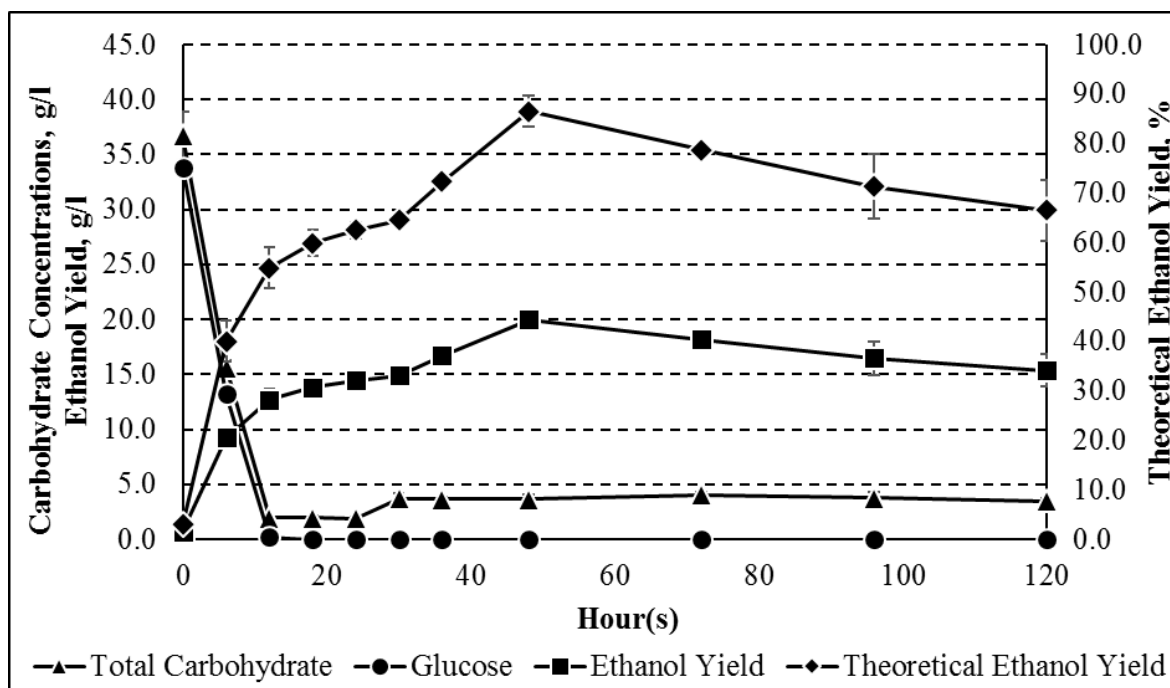


Figure 5.4: Time course of total carbohydrate, glucose residual, ethanol production with theoretical ethanol yield, as determined by HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

The concentration of other fermentation co-products such as lactic acid and acetic acid were also quantified in this study. Acetic acid production from the TSE was relatively constant throughout the SSSF period, ranging between 1.47 and 1.74 g/l (Figure 5.5). According to Vincent *et al.* (2015a), acetic acid is an important indicator to evaluate the efficiency of biomass hydrolysis, as the increase in acetic acid production depicts continuous degradation of biomass. However, as only a small amount (3.0%) of sago biomass was involved in this study, very low concentration of acetic acid was recorded. Another fermentation by-product, lactic acid, was shown to be produced immediately from the beginning of the SSSF process, similar to acetic acid. The production of lactic acid increased constantly to 2.84 g/l at 36 h, then decreased and remained relatively constant from 72 h until the end of fermentation. According to Lin *et al.* (2012), the formation of by-products may have consumed some of the substrates and reduced the efficiency of ethanol fermentation. The presence and

accumulation of these acidic by-products should be monitored constantly to control and maintain pH values in the fermentation broths to the range of 4.0-5.0 to avoid a change in the main fermentation pathway (Lin *et al*, 2012).

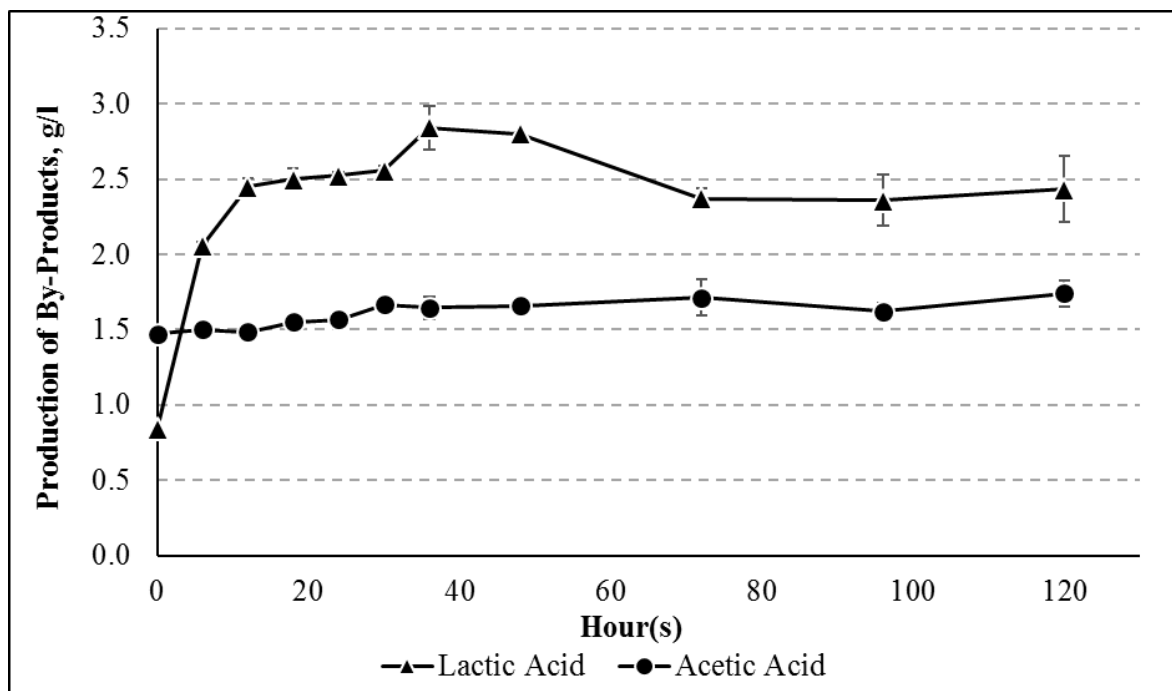


Figure 5.5: Time course of fermentation by-products, lactic acid and acetic acid productions, as determined by HPLC. [Note: The data points represent the average of three independent experiments (n=3).]

At the end of the SSSF period, solid biomass in the TSE was reduced from 6.00 to 2.10 g as shown in Figure 5.6. This indicates that $65.00 \pm 1.00\%$ of the biomass was saccharified into fermentable sugars and ultimately fermented into bioethanol. This outcome indicates that efficient utilisation of total sago effluent as bioethanol feedstock via SSSF is possible and this process may be adapted in relieving the environmental burden from the sago waste released (Linggang *et al.*, 2012).

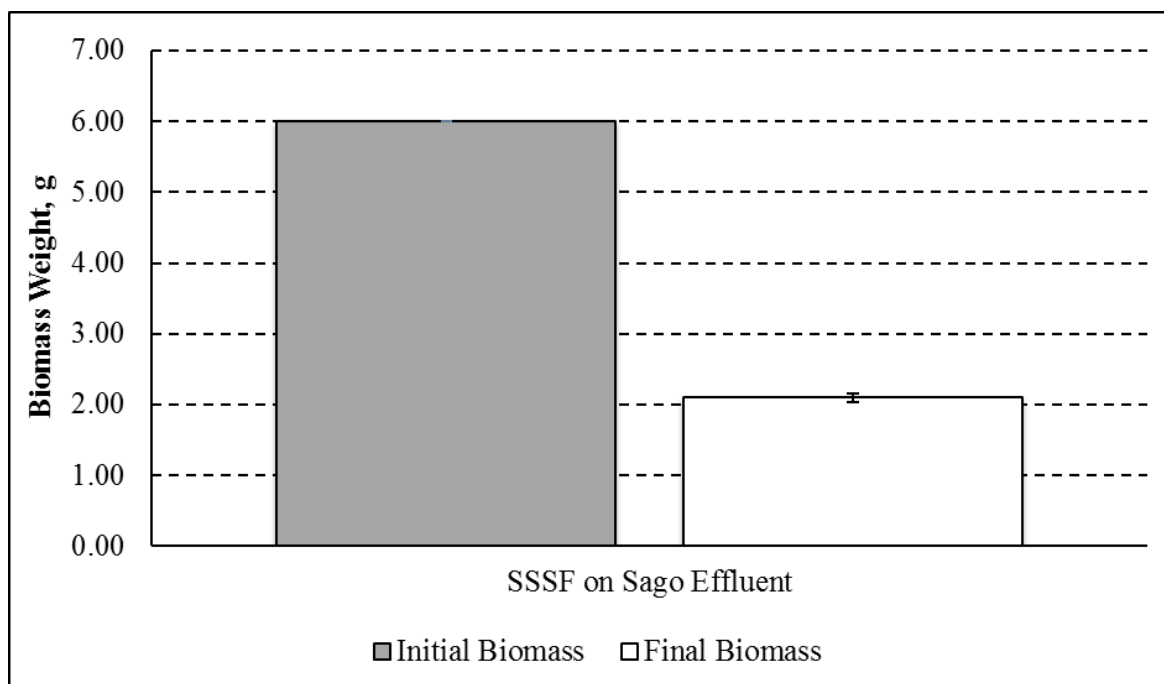


Figure 5.6: Total biomass reduction of TSE by the end of SSSF process. [Note: The data points represent the average of three independent experiments (n=3).]

5.4 Conclusion

Sequential saccharification and simultaneous fermentation (SSSF) of fresh total sago effluent (TSE) to produce ethanol was successfully performed in the current study, indicating that efficient utilisation of TSE as bioethanol feedstock via SSSF is possible. The results showed that carbohydrate-rich TSE with 36.72 ± 2.19 g/l of initial total carbohydrate was effective in generating a high ethanol yield of 20.01 ± 0.73 g/l, corresponding to $86.56 \pm 3.17\%$ theoretical ethanol yield. Hence, this process could be employed in relieving the environmental burden from the sago waste that is released into the water-bodies.

CHAPTER 6

GENERAL CONCLUSION AND RESEARCH RECOMMENDATIONS

6.1 Conclusion

Energy crisis turn out to be serious threat towards sustainability of countries worldwide due to the instabilities of fossil fuel supplies and environmental pollution. This has spawned the research interest in finding an alternative renewable energy resources such as biofuel. The production of first-generation biofuel is currently the most predominate way of producing biofuel, and this has led to a food shortage, imbalance in the food chain, increased food prices and larger land uses. An inexpensive, renewable and abundant carbohydrate-rich wastewater such as sago effluent that provides a unique natural resource is recently introduced as a feedstock of second-generation biofuel, particularly bioethanol, which is an ideal alternative to petroleum-based fuel to fulfil the increasing energy demands.

This dissertation focuses on the study of the production of bioethanol from fresh sago effluent and its constituents. The major issue that addressed in the current study was to relieve the environmental burden from the sago waste released. The other was to determine the efficiency of simultaneous saccharification and fermentation (SSF) and sequential saccharification and simultaneous fermentation (SSSF) in order to attain the highest level of ethanol productivity.

SSF was conducted on sago effluent hydrolysate and sago hampas in order to determine their potential as bioethanol production feedstocks. By performing SSF, results indicated that sago

effluent hydrolysate and sago hampas 5.0% (w/v) loading are shown to be potential bioethanol production feedstocks, producing 8.39 ± 0.20 g/l ($84.89 \pm 4.56\%$ Theoretical Ethanol Yield; TEY) and 17.79 ± 0.17 g/l ($79.65 \pm 0.78\%$ TEY) respectively. From the study using sago hampas as raw material, high carbohydrate at 7.5% (w/v) loading was inhibitor to cellulase enzyme activities in broth. Hence, SSSF was applied on carbohydrate-rich total sago effluent, where a higher ethanol production (20.01 ± 0.73 g/l), corresponding to $86.56 \pm 3.17\%$ TEY, was recorded in the samples with total sago effluent (TSE).

Based on the results from the current study, sago effluent and its constituents can be considered as potential feedstock for bioethanol production, especially in Sarawak, East Malaysia where it is available in large quantities and is relatively inexpensive.

6.2 Research Recommendations

Some criteria should be considered when sago effluent and its constituents were to be implemented in further study. Lignin has been documented to be a major hindrance to enzymatic degradation of structural carbohydrates as its close association with the microfibrils prevents enzymatic hydrolysis. In order to achieve higher yields of reducing sugar in the saccharification process, the suitability of pretreatment can be further developed and optimised in lignocellulosic ethanol production when utilising sago waste.

The strains of fermenting microorganisms are also extremely important during fermentation. Genetic engineering can be performed to construct a more compatible recombinant strain

(mixed cultures and recombinant yeast or robust strain) that is able to utilise different kind of fermentable sugars such as hexoses and pentoses, that may potentially enhance ethanol production. This is due to the fact that significant quantities of xylose and arabinose are left unutilised in hydrolysate fermented media when *Saccharomyces cerevisiae* is used as the fermenting microorganism. Hence, fermentation using genetically engineering strain of microorganisms such as *Escherichia coli* K011 can be explored and the fermentation process can be optimised for lignocellulosic biomass.

Most of the ethanol related products (beverages and biofuels) are produced via the batch system due to its low investment cost and lower risk of contamination. However, studies have shown that the ethanol yield is higher when using fed-batch culture which has the advantages of substrate reduction and end product inhibition that result in higher productivity of ethanol, decreased fermentation time and higher hydrolysis rate. Hence, a construction of mathematical modelling using fed-batch culture is suggested for further investigation of its effect on bioethanol productivity using sago effluent and its constituents as substrates.

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APPENDICES

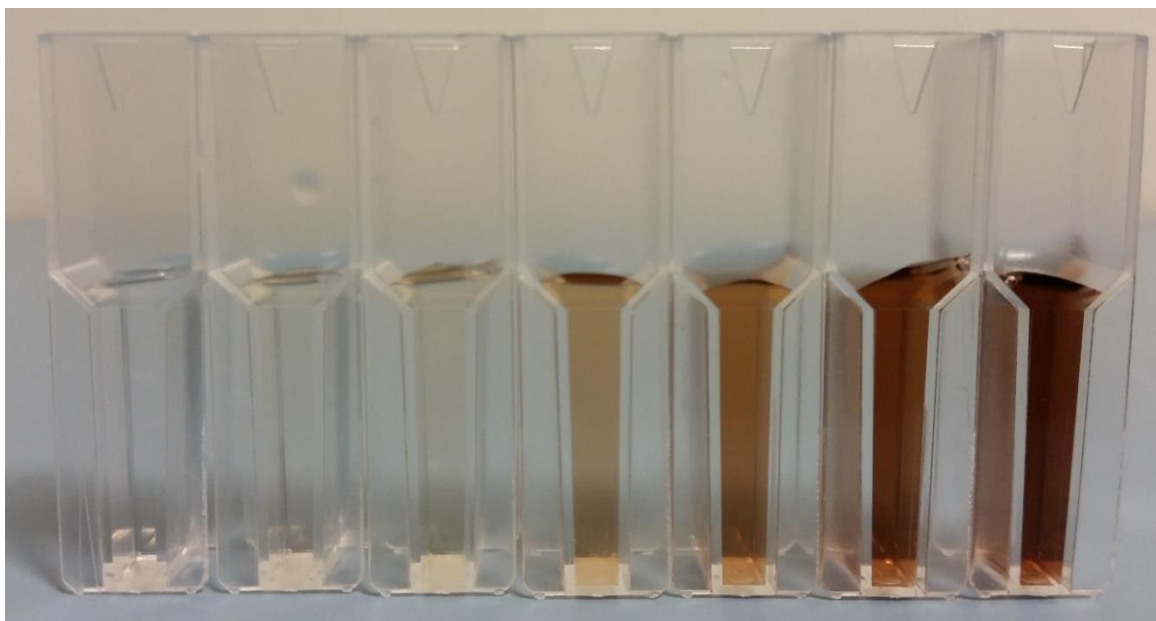


Figure 1: Colour development for standard curve of phenol-sulphuric acid assay.

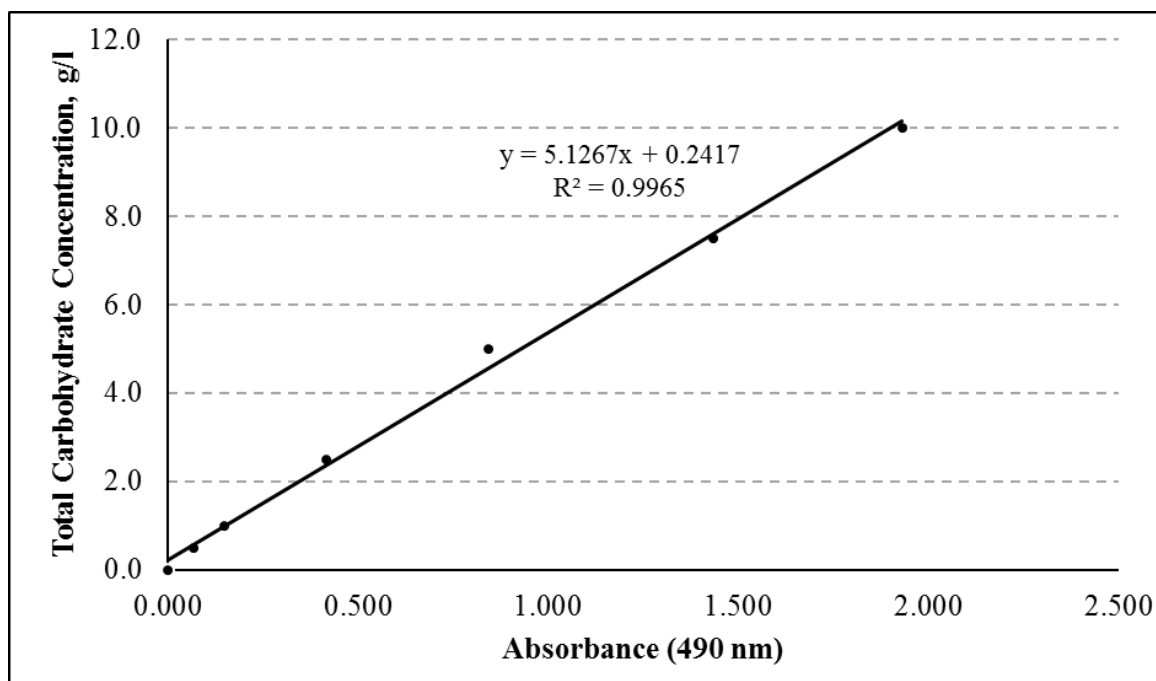
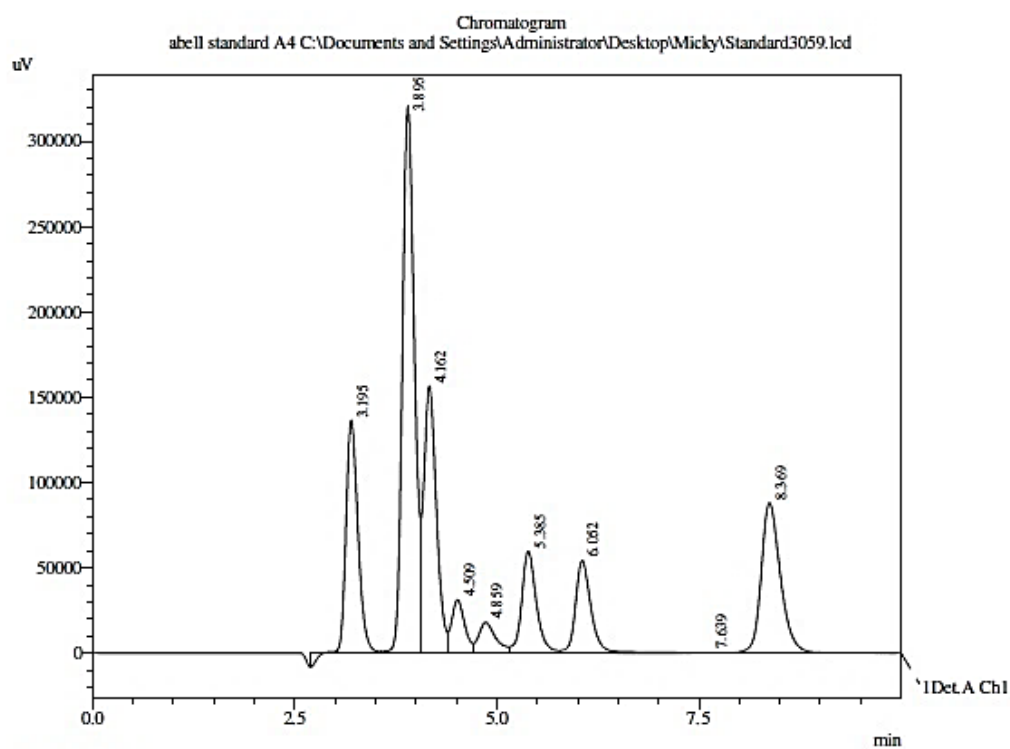


Figure 2: Standard curve for total carbohydrate concentration via phenol-sulphuric acid assay.

Sample Information

Acquired by : Admin
Sample Name : abell standard A4
Sample ID : ENNRY@BOB2016
Vial# : 1
Injection Volume : 20 uL
Data Filename : Standard3059.lcd
Method Filename : ENNRY@BOB 2015.lcm
Batch Filename :
Report Filename : MickyMay2012.lcr(Read only).lcr
Date Acquired : 5/20/2016 2:09:59 PM
Data Processed : 5/20/2016 2:21:57 PM



PeakTable

Name	Peak#	Ret. Time	Area	Height	Area %	Height %	Conc.	Units
	1	3.195	1369793	136347	13.743	15.768	0.000	
	2	3.895	3316819	320789	33.278	37.097	0.000	
	3	4.162	1702388	156472	17.080	18.095	0.000	
	4	4.509	352011	31137	3.532	3.601	0.000	
	5	4.859	264202	17903	2.651	2.070	0.000	
	6	5.385	754166	59556	7.567	6.887	0.000	
	7	6.052	737441	54279	7.399	6.277	0.000	
	8	7.639	3859	179	0.039	0.021	0.000	
	9	8.369	1466199	88059	14.711	10.183	0.000	
	Total		9966877	864721	100.000	100.000		

Figure 3: The chromatogram of High Performance Liquid Chromatography.

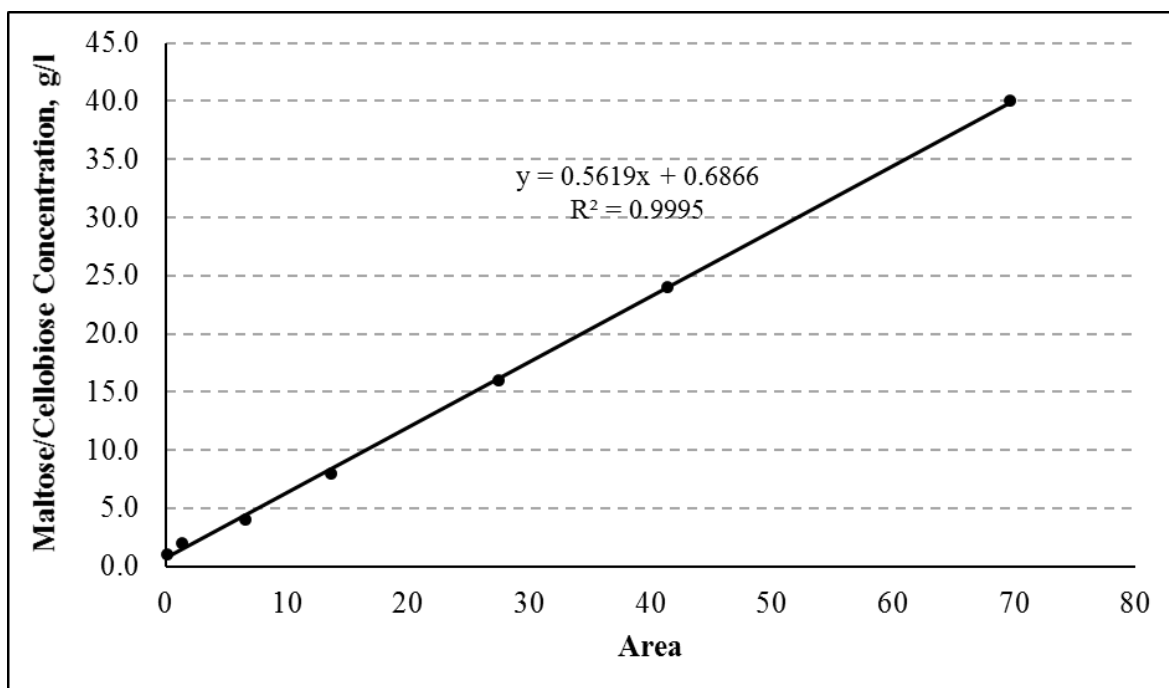


Figure 4: Standard curve for maltose/cellobiose determination via HPLC.

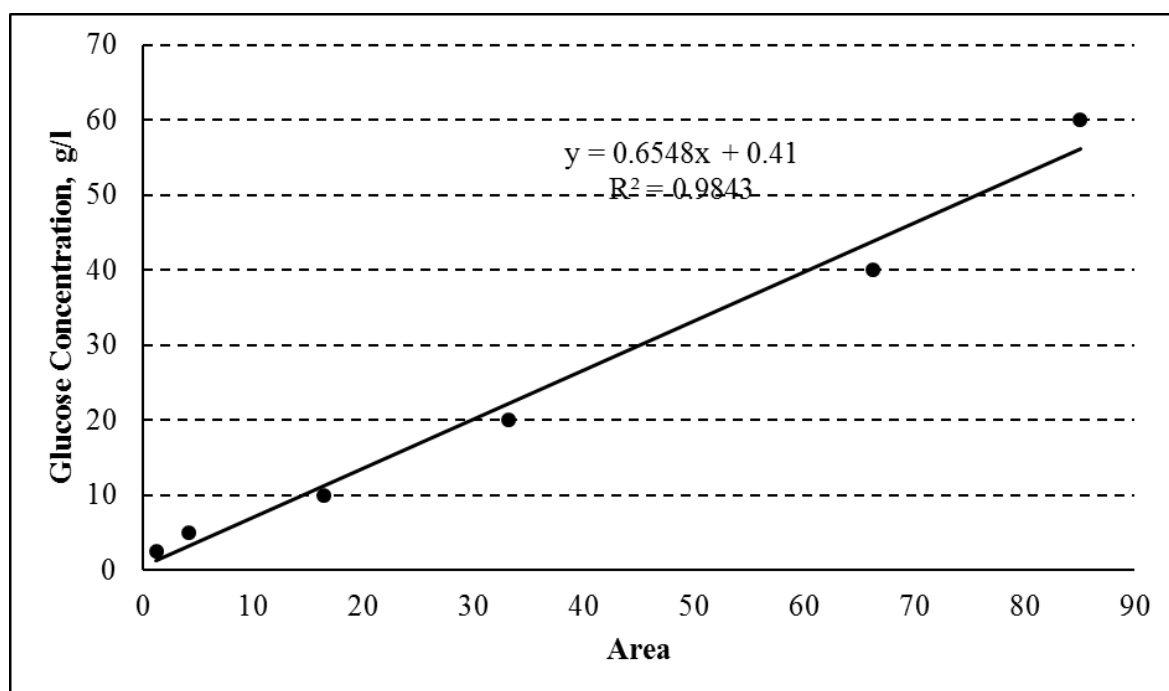


Figure 5: Standard curve for glucose determination via HPLC.

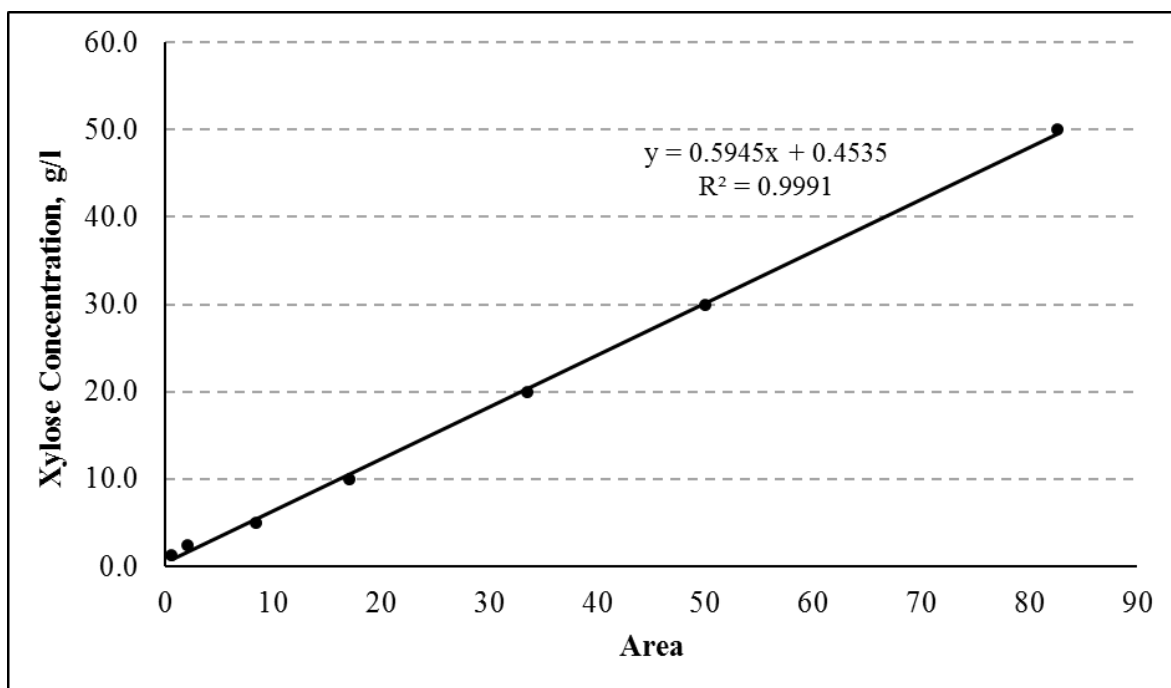


Figure 6: Standard curve for xylose determination via HPLC.

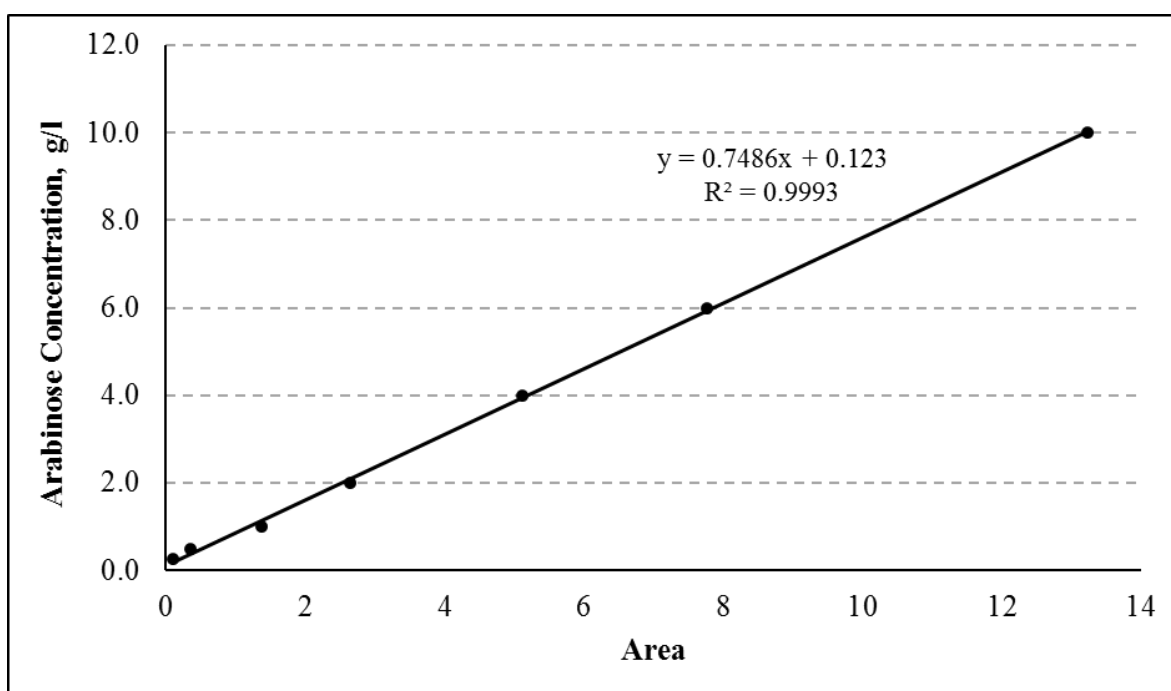


Figure 7: Standard curve for arabinose determination via HPLC.

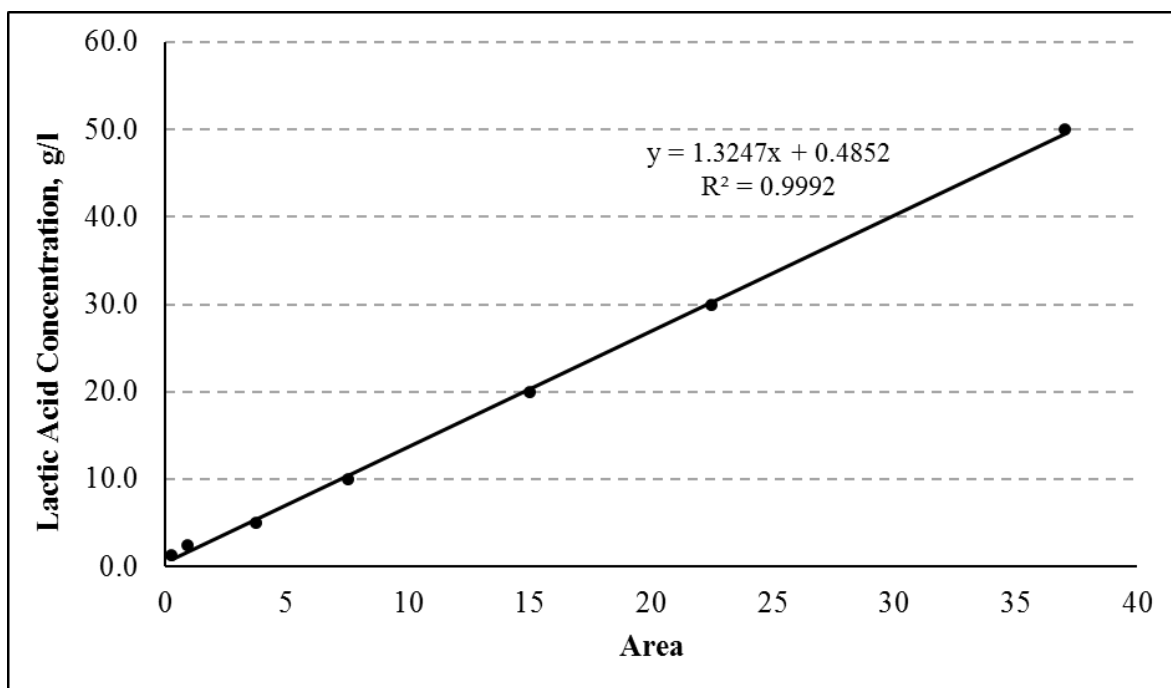


Figure 8: Standard curve for lactic acid determination via HPLC.

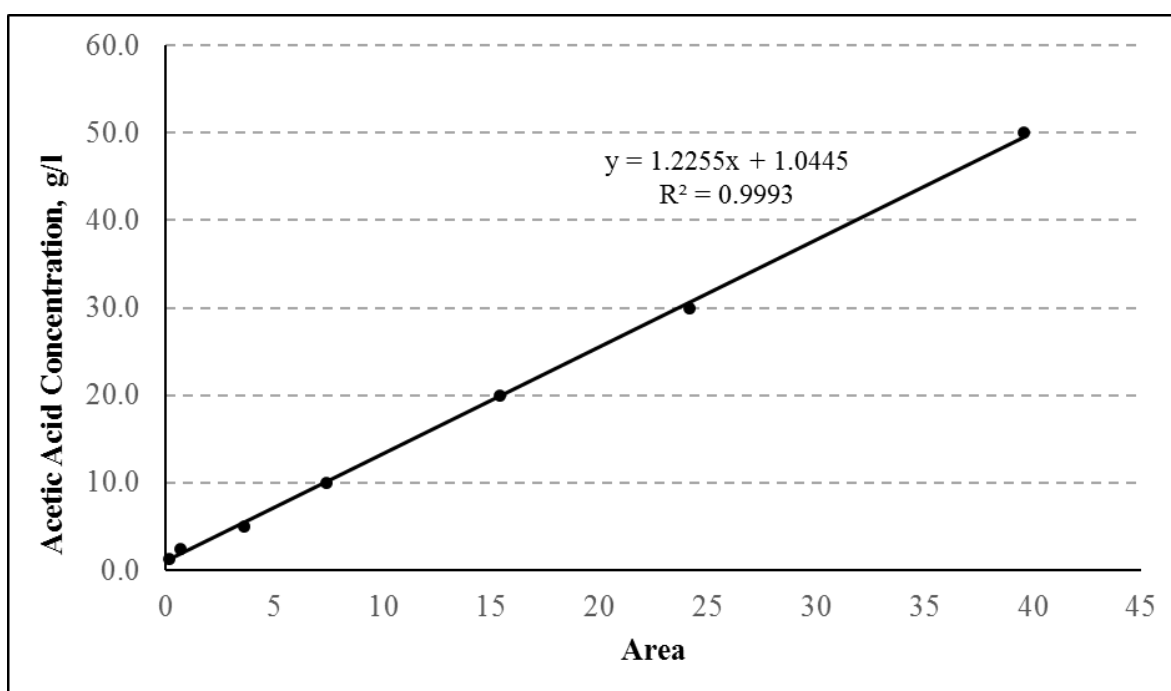


Figure 9: Standard curve for acetic acid determination via HPLC.

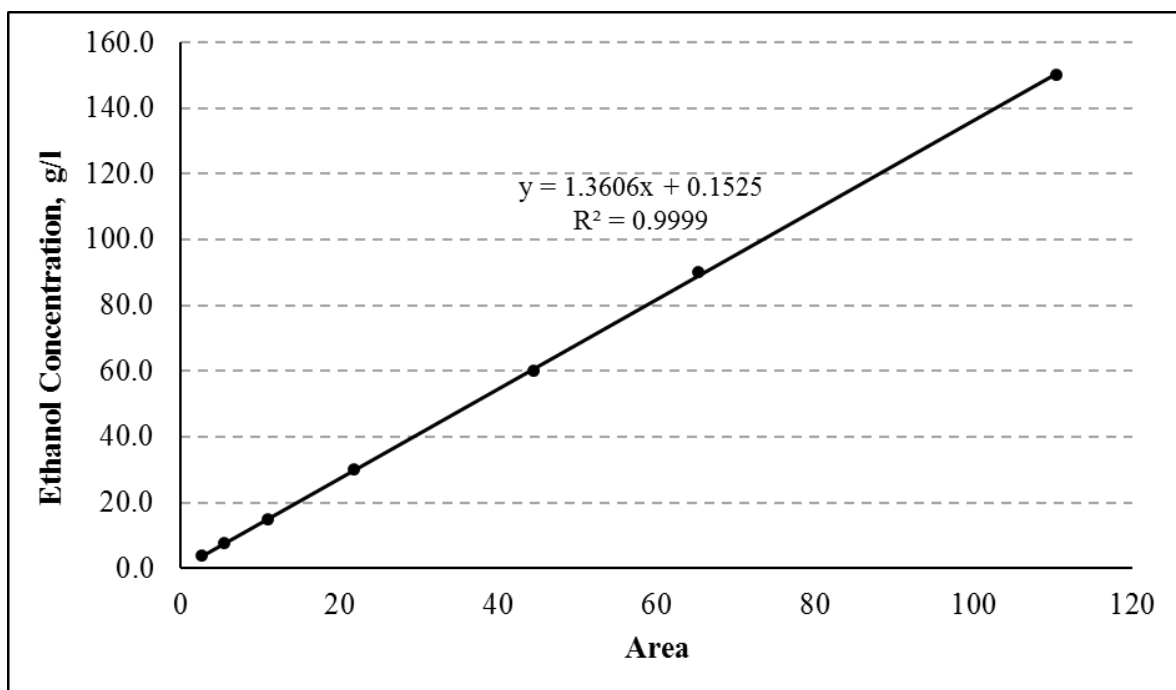


Figure 10: Standard curve for ethanol determination via HPLC.

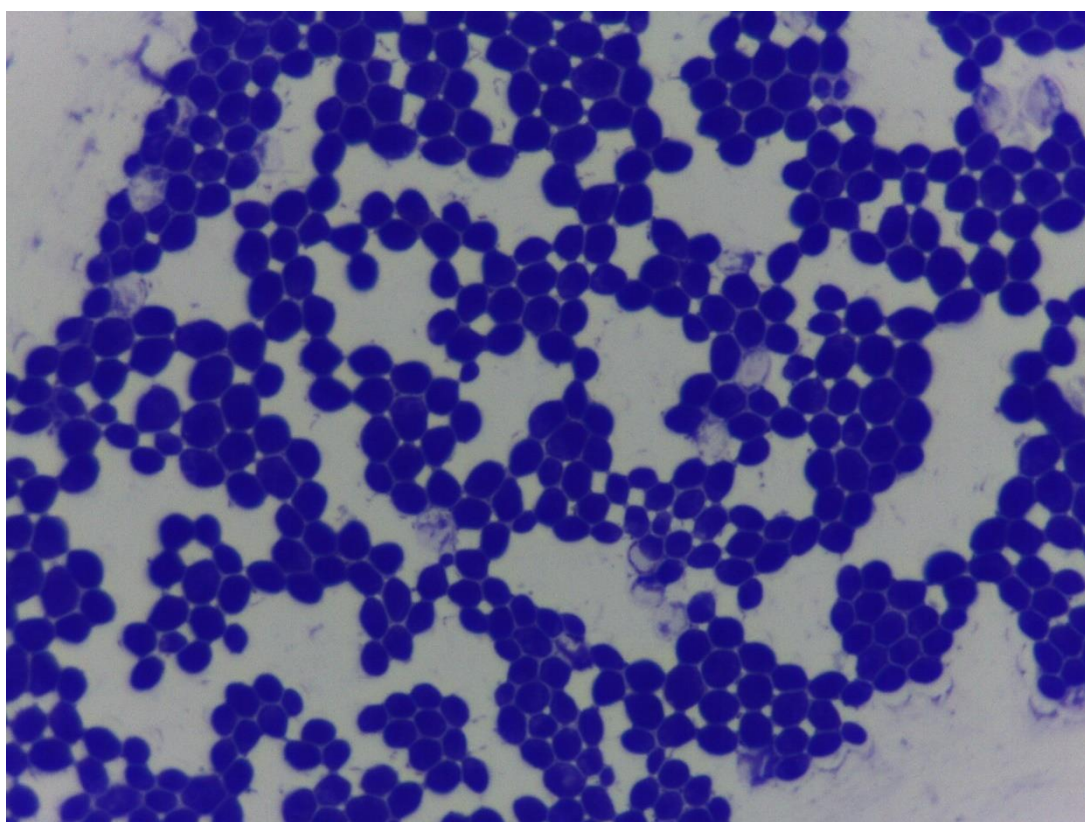


Figure 11: Morphology of *Saccharomyces cerevisiae* ATCC 24859 viewed under light microscope.

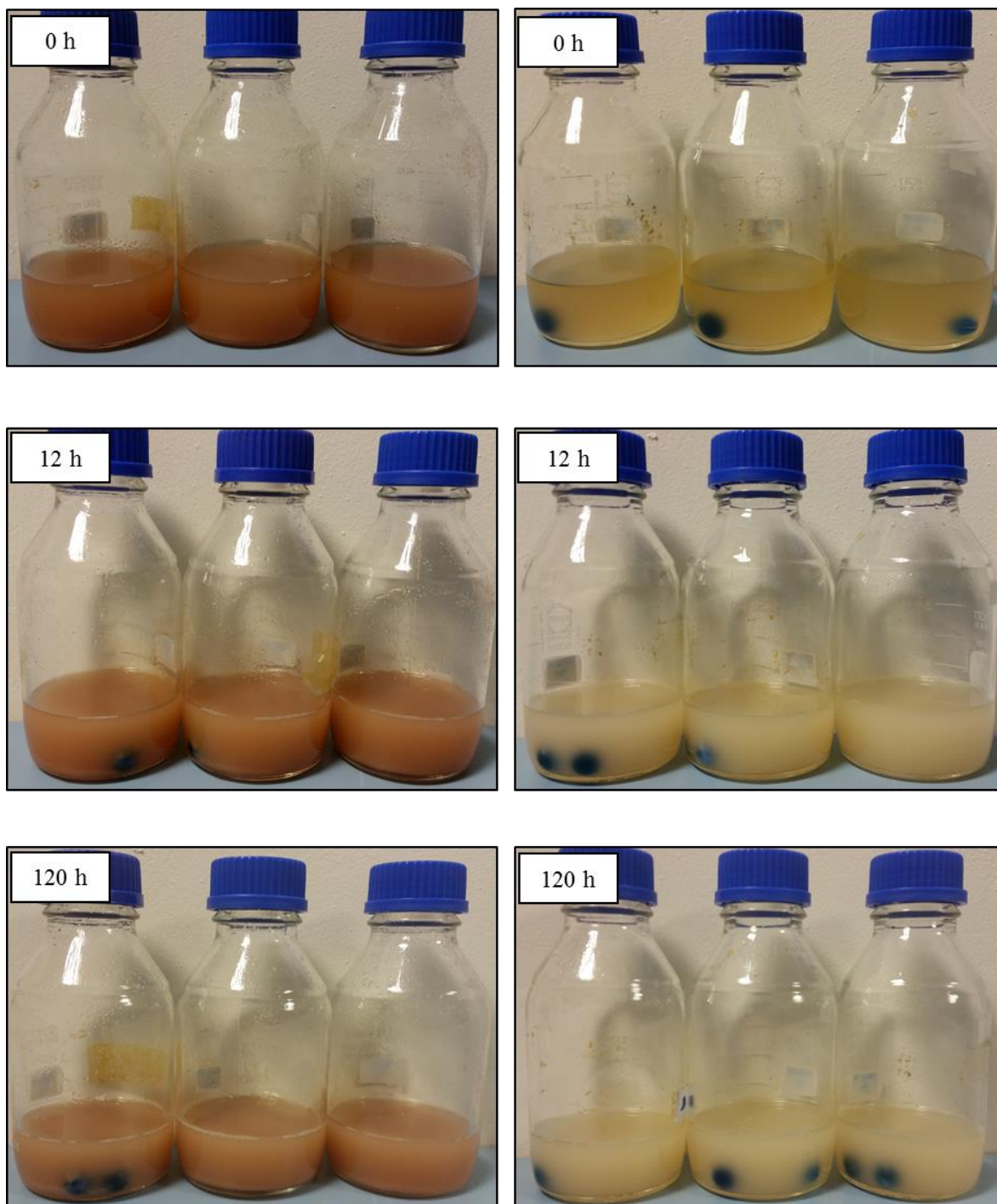


Figure 12: The fermentation broths of simultaneous saccharification and fermentation (SSF) with sago effluent hydrolysate (left); SSF with commercial starch (right) at 0 h, 12 h (highest ethanol yield) and 120 h.

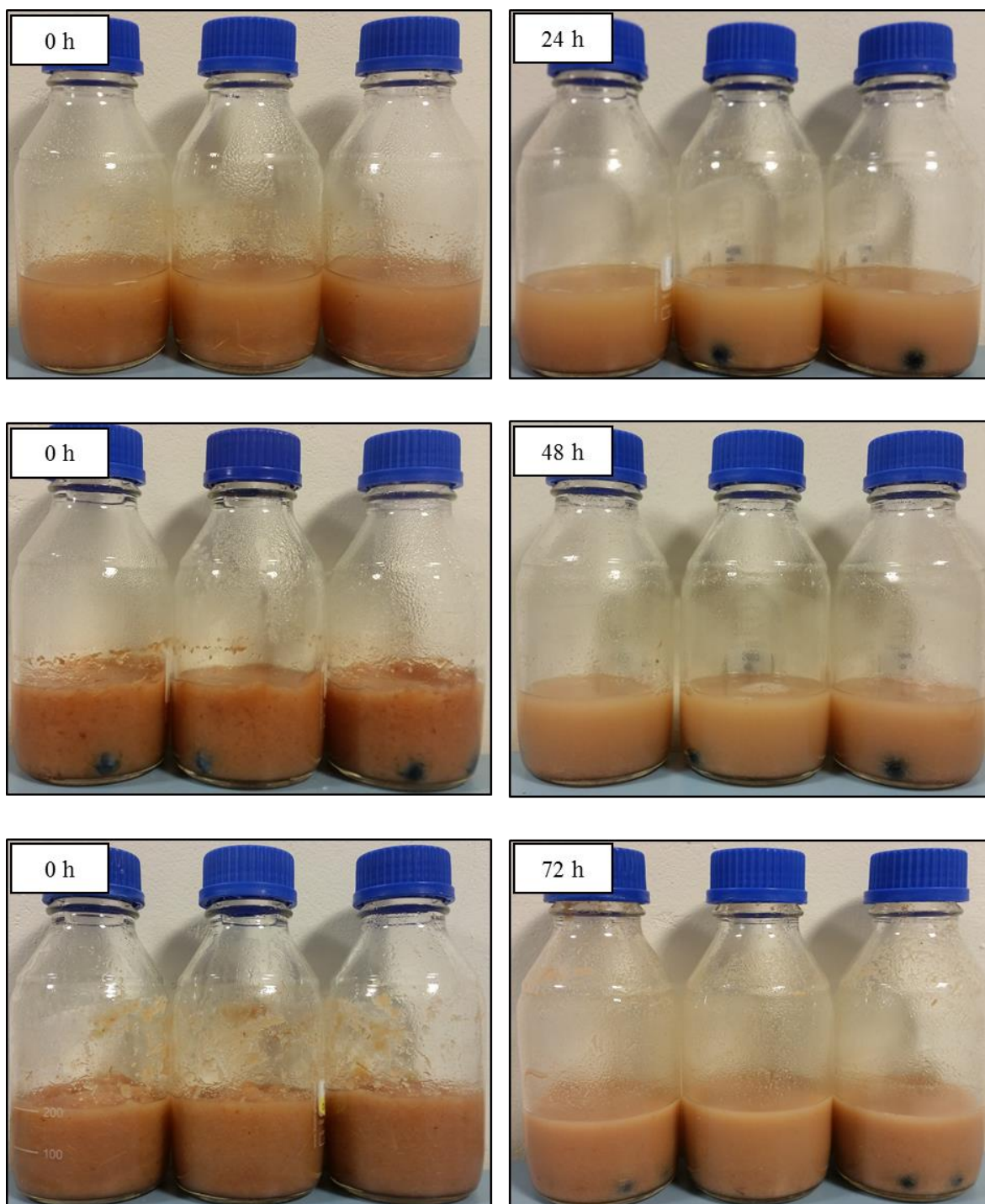


Figure 13: The fermentation broths of simultaneous saccharification and fermentation (SSF) with 2.50% (w/v) sago hampas (first row); SSF with 5.00% (w/v) sago hampas (second row); 7.50% (w/v) sago hampas at 0 h and their peak hours of highest ethanol yield.

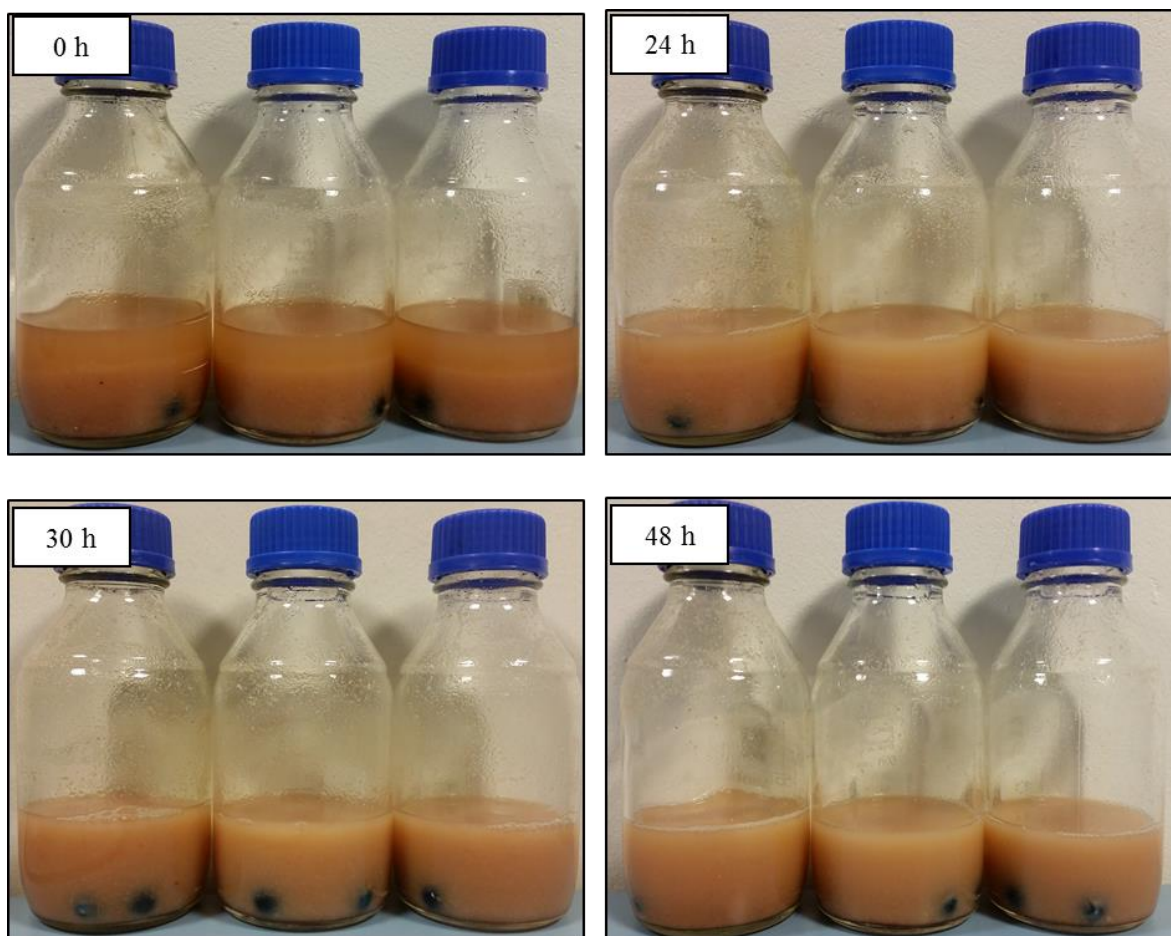


Figure 14: The fermentation broths of sequential, saccharification and simultaneous fermentation (SSSF) with total sago effluent (TSE) at the beginning of amyolytic stage (0 h), the beginning of cellulolytic stage (24 h), 30 h and its peak hour of highest ethanol production (48 h).

Table 1: Results of SSF with sago effluent hydrolysate.

Hour(s)	Total carbohydrate, g/l	Maltose, g/l	Glucose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	19.00 ± 0.51	0.94 ± 0.05	14.50 ± 0.62	0.65 ± 0.11	1.04 ± 0.09	2.27 ± 0.08	20.05 ± 0.88
6	1.98 ± 0.03	0.00 ± 0.00	0.08 ± 0.14	1.05 ± 0.03	1.09 ± 0.08	7.75 ± 0.23	76.68 ± 2.35
12	1.90 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	1.33 ± 0.08	1.12 ± 0.06	8.39 ± 0.20	84.89 ± 4.56
18	1.85 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	1.51 ± 0.08	1.13 ± 0.10	8.02 ± 0.45	79.45 ± 4.64
24	1.86 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	1.58 ± 0.07	1.13 ± 0.02	7.70 ± 0.11	76.22 ± 1.16
30	1.80 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	1.56 ± 0.05	1.09 ± 0.04	7.45 ± 0.46	73.59 ± 4.70
36	1.75 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	1.49 ± 0.06	1.06 ± 0.10	7.47 ± 0.29	73.83 ± 2.97
48	1.79 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	1.50 ± 0.06	0.96 ± 0.11	7.41 ± 0.39	73.18 ± 4.02
72	1.78 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	1.36 ± 0.25	0.96 ± 0.07	7.28 ± 0.42	71.82 ± 4.36
96	1.71 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	1.34 ± 0.26	0.89 ± 0.08	7.23 ± 0.49	71.33 ± 5.06
120	1.57 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	1.22 ± 0.35	0.85 ± 0.04	6.07 ± 0.19	62.84 ± 7.50

Table 2: Results of SSF with commercial starch.

Hour(s)	Total carbohydrate, g/l	Maltose, g/l	Glucose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	19.00 ± 0.13	1.07 ± 0.02	17.66 ± 0.33	0.55 ± 0.04	0.82 ± 0.02	1.51 ± 0.02	15.57 ± 0.22
6	0.83 ± 0.06	0.00 ± 0.00	0.37 ± 0.06	2.16 ± 0.03	0.97 ± 0.01	8.73 ± 0.05	89.91 ± 0.56
12	0.75 ± 0.12	0.00 ± 0.00	0.19 ± 0.04	2.19 ± 0.02	1.01 ± 0.03	8.88 ± 0.07	91.49 ± 0.74
18	0.69 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	2.14 ± 0.02	1.01 ± 0.01	8.69 ± 0.06	89.50 ± 0.64
24	0.60 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	2.13 ± 0.03	1.01 ± 0.01	8.58 ± 0.09	88.35 ± 0.98
30	0.62 ± 0.06	0.00 ± 0.00	0.07 ± 0.12	2.14 ± 0.07	1.01 ± 0.06	8.60 ± 0.07	88.56 ± 0.71
36	0.68 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	2.11 ± 0.05	0.96 ± 0.04	8.55 ± 0.14	88.06 ± 1.43
48	0.57 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	2.15 ± 0.04	0.93 ± 0.04	8.38 ± 0.15	86.35 ± 1.59
72	0.53 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	2.13 ± 0.04	0.94 ± 0.04	8.30 ± 0.08	85.44 ± 0.83
96	0.60 ± 0.06	0.00 ± 0.00	0.09 ± 0.15	2.18 ± 0.07	0.98 ± 0.09	8.23 ± 0.50	84.81 ± 5.15
120	0.61 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	1.93 ± 0.19	0.91 ± 0.01	6.86 ± 0.07	70.65 ± 0.68

Table 4: Results of SSF with 2.5% sago hampas loading.

Hour(s)	Total carbohydrate, g/l	Maltose/cellobiose, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	18.26 ± 0.32	1.03 ± 0.08	18.29 ± 0.51	0.00 ± 0.00	0.25 ± 0.02	0.81 ± 0.04	1.19 ± 0.05	0.33 ± 0.04	2.91 ± 0.35
6	5.01 ± 0.16	0.00 ± 0.00	1.35 ± 0.17	0.63 ± 0.03	0.36 ± 0.03	1.68 ± 0.08	1.30 ± 0.03	6.64 ± 0.17	59.44 ± 1.48
12	4.35 ± 0.27	0.00 ± 0.00	0.00 ± 0.00	0.69 ± 0.04	0.42 ± 0.04	1.81 ± 0.06	1.43 ± 0.03	6.95 ± 0.55	62.22 ± 4.92
18	4.23 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.69 ± 0.02	0.44 ± 0.02	1.82 ± 0.04	1.48 ± 0.04	7.69 ± 0.62	68.85 ± 5.54
24	4.22 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	0.72 ± 0.03	0.46 ± 0.03	1.82 ± 0.02	1.49 ± 0.01	8.38 ± 0.07	75.00 ± 0.63
30	4.12 ± 0.18	0.00 ± 0.00	0.00 ± 0.00	0.74 ± 0.06	0.52 ± 0.05	1.86 ± 0.01	1.48 ± 0.02	7.49 ± 0.32	67.05 ± 2.89
36	4.11 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.74 ± 0.06	0.52 ± 0.04	1.83 ± 0.03	1.46 ± 0.03	6.49 ± 0.58	58.15 ± 5.17
48	3.79 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	0.78 ± 0.08	0.58 ± 0.06	1.81 ± 0.06	1.46 ± 0.00	6.60 ± 0.46	59.11 ± 4.02
72	3.90 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.82 ± 0.09	0.66 ± 0.05	1.80 ± 0.01	1.44 ± 0.01	6.78 ± 0.22	60.74 ± 1.93
96	3.71 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.83 ± 0.10	0.70 ± 0.03	1.81 ± 0.04	1.45 ± 0.04	7.06 ± 0.09	63.20 ± 0.78
120	3.59 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.83 ± 0.09	0.72 ± 0.01	1.78 ± 0.06	1.43 ± 0.05	6.97 ± 0.27	62.38 ± 2.43

Table 4: Results of SSF with 5.0% sago hampas loading.

Hour(s)	Total carbohydrate, g/l	Maltose/cellobiose, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	39.55 ± 0.10	1.27 ± 0.13	34.72 ± 1.72	0.00 ± 0.00	0.28 ± 0.01	0.78 ± 0.06	1.24 ± 0.03	0.46 ± 0.05	2.08 ± 0.24
6	22.96 ± 0.38	2.22 ± 0.14	15.65 ± 0.94	0.00 ± 0.00	0.40 ± 0.01	2.23 ± 0.06	1.43 ± 0.01	9.68 ± 0.23	43.35 ± 1.01
12	7.19 ± 0.10	0.00 ± 0.00	0.87 ± 0.06	2.88 ± 0.03	0.51 ± 0.01	2.75 ± 0.05	1.45 ± 0.03	16.13 ± 0.31	72.22 ± 1.40
18	7.06 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	2.91 ± 0.06	0.59 ± 0.02	2.86 ± 0.09	1.57 ± 0.03	17.29 ± 0.44	77.41 ± 1.98
24	6.92 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	2.88 ± 0.03	0.60 ± 0.01	2.84 ± 0.09	1.60 ± 0.04	17.20 ± 0.45	76.98 ± 2.02
30	7.08 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	2.90 ± 0.03	0.63 ± 0.01	2.86 ± 0.06	1.63 ± 0.03	17.35 ± 0.35	77.66 ± 1.56
36	6.83 ± 0.28	0.00 ± 0.00	0.00 ± 0.00	2.96 ± 0.05	0.68 ± 0.02	2.91 ± 0.09	1.65 ± 0.03	17.03 ± 0.15	76.23 ± 0.67
48	6.58 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	3.14 ± 0.04	0.76 ± 0.07	2.98 ± 0.03	1.71 ± 0.07	17.79 ± 0.17	79.65 ± 0.78
72	6.94 ± 0.62	0.00 ± 0.00	0.00 ± 0.00	3.27 ± 0.04	0.76 ± 0.04	2.89 ± 0.07	1.78 ± 0.04	17.16 ± 0.31	76.81 ± 1.40
96	5.98 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	3.34 ± 0.14	0.75 ± 0.01	2.94 ± 0.06	1.77 ± 0.04	16.50 ± 0.82	73.88 ± 3.68
120	6.24 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	3.44 ± 0.03	0.61 ± 0.08	2.85 ± 0.06	1.85 ± 0.06	14.09 ± 0.42	63.06 ± 1.87

Table 5: Results of SSF with 7.5% sago hampas loading.

Hour(s)	Total carbohydrate, g/l	Maltose/cellobiose, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	56.53 ± 1.04	1.54 ± 0.06	42.83 ± 1.63	0.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.05	1.29 ± 0.01	0.45 ± 0.07	1.35 ± 0.21
6	37.29 ± 0.27	2.74 ± 0.03	32.28 ± 2.93	0.00 ± 0.00	0.31 ± 0.27	2.10 ± 0.23	1.50 ± 0.01	7.26 ± 1.35	21.68 ± 4.02
12	9.63 ± 0.30	0.00 ± 0.00	5.74 ± 8.18	3.80 ± 0.28	0.55 ± 0.09	3.32 ± 0.06	1.74 ± 0.13	20.70 ± 0.66	61.77 ± 1.98
18	8.49 ± 0.59	0.00 ± 0.00	0.00 ± 0.00	3.65 ± 0.01	0.68 ± 0.03	3.39 ± 0.23	1.77 ± 0.07	21.10 ± 1.26	62.97 ± 3.77
24	8.77 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	3.75 ± 0.20	0.72 ± 0.03	3.52 ± 0.20	1.79 ± 0.06	21.39 ± 1.73	63.82 ± 5.15
30	8.52 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	3.85 ± 0.22	0.77 ± 0.03	3.55 ± 0.21	1.82 ± 0.01	22.17 ± 0.20	66.16 ± 0.59
36	8.38 ± 1.02	0.00 ± 0.00	0.00 ± 0.00	3.73 ± 0.48	0.79 ± 0.10	3.32 ± 0.23	1.82 ± 0.08	22.57 ± 0.30	67.35 ± 0.89
48	8.31 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	3.87 ± 0.30	0.88 ± 0.04	3.50 ± 0.21	1.91 ± 0.04	22.48 ± 0.09	67.08 ± 0.27
72	8.45 ± 0.78	0.00 ± 0.00	0.00 ± 0.00	4.08 ± 0.08	0.90 ± 0.14	3.65 ± 0.15	2.07 ± 0.05	23.28 ± 0.61	69.48 ± 1.81
96	7.80 ± 0.47	0.00 ± 0.00	0.00 ± 0.00	4.21 ± 0.05	1.11 ± 0.03	3.65 ± 0.16	2.01 ± 0.09	22.41 ± 0.14	66.87 ± 0.43
120	7.37 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	4.38 ± 0.08	1.14 ± 0.08	3.69 ± 0.15	2.07 ± 0.08	22.28 ± 0.48	66.49 ± 1.43

Table 6: Results of SSSF with total sago effluent.

Hour(s)	Total carbohydrate, g/l	Maltose/cellobiose, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	36.72 ± 2.19	1.01 ± 0.06	33.86 ± 1.86	0.00 ± 0.00	0.23 ± 0.01	0.84 ± 0.02	1.47 ± 0.01	0.69 ± 0.05	3.00 ± 0.21
6	15.63 ± 0.68	1.00 ± 0.02	13.18 ± 4.13	0.00 ± 0.00	0.35 ± 0.03	2.06 ± 0.03	1.50 ± 0.02	9.26 ± 0.97	40.08 ± 4.19
12	1.97 ± 0.11	0.83 ± 0.02	0.21 ± 0.37	0.00 ± 0.00	0.40 ± 0.01	2.45 ± 0.06	1.49 ± 0.05	12.69 ± 0.96	54.90 ± 4.17
18	1.96 ± 0.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.41 ± 0.02	2.50 ± 0.07	1.55 ± 0.01	13.85 ± 0.59	59.93 ± 2.55
24	1.91 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.05	2.53 ± 0.02	1.57 ± 0.04	14.45 ± 0.38	62.54 ± 1.66
30	3.72 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.46 ± 0.04	2.56 ± 0.03	1.67 ± 0.03	14.90 ± 0.26	64.46 ± 1.13
36	3.65 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.76 ± 0.01	0.48 ± 0.08	2.84 ± 0.15	1.65 ± 0.07	16.70 ± 0.18	72.26 ± 0.76
48	3.66 ± 0.41	0.00 ± 0.00	0.00 ± 0.00	0.73 ± 0.02	0.59 ± 0.01	2.80 ± 0.02	1.66 ± 0.01	20.01 ± 0.73	86.56 ± 3.17
72	4.03 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.80 ± 0.03	0.68 ± 0.04	2.37 ± 0.07	1.71 ± 0.12	18.18 ± 0.08	78.66 ± 0.37
96	3.78 ± 0.53	0.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.02	0.67 ± 0.01	2.36 ± 0.17	1.62 ± 0.06	16.48 ± 1.51	71.30 ± 6.55
120	3.50 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.06	0.73 ± 0.07	2.43 ± 0.22	1.74 ± 0.09	15.36 ± 1.43	66.48 ± 6.21

Table 4: Results of SSF with 5.00% sago hampas loading.

Hour(s)	Total carbohydrate, g/l	Maltose/cellobiose, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	39.55 ± 0.10	1.27 ± 0.13	34.72 ± 1.72	0.00 ± 0.00	0.28 ± 0.01	0.78 ± 0.06	1.24 ± 0.03	0.46 ± 0.05	2.08 ± 0.24
6	22.96 ± 0.38	2.22 ± 0.14	15.65 ± 0.94	0.00 ± 0.00	0.40 ± 0.01	2.23 ± 0.06	1.43 ± 0.01	9.68 ± 0.23	43.35 ± 1.01
12	7.19 ± 0.10	0.00 ± 0.00	0.87 ± 0.06	2.88 ± 0.03	0.51 ± 0.01	2.75 ± 0.05	1.45 ± 0.03	16.13 ± 0.31	72.22 ± 1.40
18	7.06 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	2.91 ± 0.06	0.59 ± 0.02	2.86 ± 0.09	1.57 ± 0.03	17.29 ± 0.44	77.41 ± 1.98
24	6.92 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	2.88 ± 0.03	0.60 ± 0.01	2.84 ± 0.09	1.60 ± 0.04	17.20 ± 0.45	76.98 ± 2.02
30	7.08 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	2.90 ± 0.03	0.63 ± 0.01	2.86 ± 0.06	1.63 ± 0.03	17.35 ± 0.35	77.66 ± 1.56
36	6.83 ± 0.28	0.00 ± 0.00	0.00 ± 0.00	2.96 ± 0.05	0.68 ± 0.02	2.91 ± 0.09	1.65 ± 0.03	17.03 ± 0.15	76.23 ± 0.67
48	6.58 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	3.14 ± 0.04	0.76 ± 0.07	2.98 ± 0.03	1.71 ± 0.07	17.79 ± 0.17	79.65 ± 0.78
72	6.94 ± 0.62	0.00 ± 0.00	0.00 ± 0.00	3.27 ± 0.04	0.76 ± 0.04	2.89 ± 0.07	1.78 ± 0.04	17.16 ± 0.31	76.81 ± 1.40
96	5.98 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	3.34 ± 0.14	0.75 ± 0.01	2.94 ± 0.06	1.77 ± 0.04	16.50 ± 0.82	73.88 ± 3.68
120	6.24 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	3.44 ± 0.03	0.61 ± 0.08	2.85 ± 0.06	1.85 ± 0.06	14.09 ± 0.42	63.06 ± 1.87

Table 5: Results of SSF with 7.50% sago hampas loading.

Hour(s)	Total carbohydrate, g/l	Maltose/cellobiose, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	56.53 ± 1.04	1.54 ± 0.06	42.83 ± 1.63	0.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.05	1.29 ± 0.01	0.45 ± 0.07	1.35 ± 0.21
6	37.29 ± 0.27	2.74 ± 0.03	32.28 ± 2.93	0.00 ± 0.00	0.31 ± 0.27	2.10 ± 0.23	1.50 ± 0.01	7.26 ± 1.35	21.68 ± 4.02
12	9.63 ± 0.30	0.00 ± 0.00	5.74 ± 8.18	3.80 ± 0.28	0.55 ± 0.09	3.32 ± 0.06	1.74 ± 0.13	20.70 ± 0.66	61.77 ± 1.98
18	8.49 ± 0.59	0.00 ± 0.00	0.00 ± 0.00	3.65 ± 0.01	0.68 ± 0.03	3.39 ± 0.23	1.77 ± 0.07	21.10 ± 1.26	62.97 ± 3.77
24	8.77 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	3.75 ± 0.20	0.72 ± 0.03	3.52 ± 0.20	1.79 ± 0.06	21.39 ± 1.73	63.82 ± 5.15
30	8.52 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	3.85 ± 0.22	0.77 ± 0.03	3.55 ± 0.21	1.82 ± 0.01	22.17 ± 0.20	66.16 ± 0.59
36	8.38 ± 1.02	0.00 ± 0.00	0.00 ± 0.00	3.73 ± 0.48	0.79 ± 0.10	3.32 ± 0.23	1.82 ± 0.08	22.57 ± 0.30	67.35 ± 0.89
48	8.31 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	3.87 ± 0.30	0.88 ± 0.04	3.50 ± 0.21	1.91 ± 0.04	22.48 ± 0.09	67.08 ± 0.27
72	8.45 ± 0.78	0.00 ± 0.00	0.00 ± 0.00	4.08 ± 0.08	0.90 ± 0.14	3.65 ± 0.15	2.07 ± 0.05	23.28 ± 0.61	69.48 ± 1.81
96	7.80 ± 0.47	0.00 ± 0.00	0.00 ± 0.00	4.21 ± 0.05	1.11 ± 0.03	3.65 ± 0.16	2.01 ± 0.09	22.41 ± 0.14	66.87 ± 0.43
120	7.37 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	4.38 ± 0.08	1.14 ± 0.08	3.69 ± 0.15	2.07 ± 0.08	22.28 ± 0.48	66.49 ± 1.43

Table 6: Results of SSSF with total sago effluent.

Hour(s)	Total carbohydrate, g/l	Maltose/cellobiose, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l	Lactic acid, g/l	Acetic acid, g/l	Ethanol, g/l	TEY, %
0	36.72 ± 2.19	1.01 ± 0.06	33.86 ± 1.86	0.00 ± 0.00	0.23 ± 0.01	0.84 ± 0.02	1.47 ± 0.01	0.69 ± 0.05	3.00 ± 0.21
6	15.63 ± 0.68	1.00 ± 0.02	13.18 ± 4.13	0.00 ± 0.00	0.35 ± 0.03	2.06 ± 0.03	1.50 ± 0.02	9.26 ± 0.97	40.08 ± 4.19
12	1.97 ± 0.11	0.83 ± 0.02	0.21 ± 0.37	0.00 ± 0.00	0.40 ± 0.01	2.45 ± 0.06	1.49 ± 0.05	12.69 ± 0.96	54.90 ± 4.17
18	1.96 ± 0.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.41 ± 0.02	2.50 ± 0.07	1.55 ± 0.01	13.85 ± 0.59	59.93 ± 2.55
24	1.91 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.05	2.53 ± 0.02	1.57 ± 0.04	14.45 ± 0.38	62.54 ± 1.66
30	3.72 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.46 ± 0.04	2.56 ± 0.03	1.67 ± 0.03	14.90 ± 0.26	64.46 ± 1.13
36	3.65 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.76 ± 0.01	0.48 ± 0.08	2.84 ± 0.15	1.65 ± 0.07	16.70 ± 0.18	72.26 ± 0.76
48	3.66 ± 0.41	0.00 ± 0.00	0.00 ± 0.00	0.73 ± 0.02	0.59 ± 0.01	2.80 ± 0.02	1.66 ± 0.01	20.01 ± 0.73	86.56 ± 3.17
72	4.03 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.80 ± 0.03	0.68 ± 0.04	2.37 ± 0.07	1.71 ± 0.12	18.18 ± 0.08	78.66 ± 0.37
96	3.78 ± 0.53	0.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.02	0.67 ± 0.01	2.36 ± 0.17	1.62 ± 0.06	16.48 ± 1.51	71.30 ± 6.55
120	3.50 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.06	0.73 ± 0.07	2.43 ± 0.22	1.74 ± 0.09	15.36 ± 1.43	66.48 ± 6.21

Table 7: Summary of univariate analysis of variance for sago effluent hydrolysate and commercial starch.

Effect	Feedstock	Mean	Standard Deviation	R squared	F	Sig.
Initial carbohydrate	Sago effluent hydrolysate	19.0007	0.50522	0.000	0.000	0.998
	Commercial starch	19.0000	0.12835			
Initial glucose	Sago effluent hydrolysate	14.5013	0.61880	0.938	60.893	0.001
	Commercial starch	17.6617	0.33038			
Lactic acid	Sago effluent hydrolysate	1.3310	0.08021	0.988	325.240	0.000
	Commercial starch	2.1913	0.01986			
Acetic acid	Sago effluent hydrolysate	1.1223	0.05757	0.670	8.135	0.046
	Commercial starch	1.0147	0.03099			
Ethanol yield	Sago effluent hydrolysate	8.5413	0.44198	0.304	1.746	0.257
	Commercial starch	8.8830	0.07202			

Table 8: *Post hoc* tests between three independent experiments of sago hampas loadings.

Aspect	(I) Feedstocks	(J) Feedstocks	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Ethanol production	2.50% Sago hampas loading	5.00% Sago hampas loading	-8.1200	1.30253	0.000	-11.2208	-5.0192
		7.50% Sago hampas loading	-12.2464	1.30253	0.000	-15.3472	-9.1456
	5.00% Sago hampas loading	2.50% Sago hampas loading	8.1200	1.30253	0.000	5.0192	11.2208
		7.50% Sago hampas loading	-4.1264	1.30253	0.000	-7.2272	-1.0256
	7.50% Sago hampas loading	2.50% Sago hampas loading	12.2464	1.30253	0.000	9.1456	15.3472
		5.00% Sago hampas loading	4.1264	1.30253	0.000	1.0256	7.2272