



**Faculty of Resource Science and Technology**

**Evaluation of *Candida glabrata* for Simultaneous Saccharification and Fermentation (SSF) of Total Sago Effluent**

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Evaluation of *Candida glabrata* for Simultaneous Saccharification and  
Fermentation (SSF) of Total Sago Effluent

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A thesis submitted

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## DECLARATION

I hereby declare that the dissertation entitled, “Evaluation of *Candida glabrata* for Simultaneous Saccharification and Fermentation (SSF) of Total 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 degree.

.....

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## ABSTRACT

Sago palm plantations are mostly found in Sarawak and the sago industries have produced tons of wastes every year. These sago wastes could be potential biomass substrate for bioethanol production as it can be found abundantly. In bioethanol production, the ability of microorganisms to ferment substrate is important to achieve high ethanol yield. *Candida glabrata* is commonly found in fermented foods such as *ragi* and can potentially be used as a fermentative organism in bioethanol production. Hence, in this study, *C. glabrata* was evaluated to convert carbohydrate substrates to ethanol. The first objective of the study was evaluation of *C. glabrata* in production of ethanol from simple sugar such as glucose, maltose and sucrose. Fermentation process was carried out for 5 days, with 150 rpm at ambient temperature. Results indicated that *C. glabrata* only consumed glucose and sucrose with the maximum ethanol production of approximately 20.4 g/l and 1.64 g/l, respectively. Among these sugars, *C. glabrata* yielded the highest ethanol content in glucose substrate. This showed that *C. glabrata* could be a potential fermentative organism since it can ferment glucose efficiently. Next, the fermenting capability of *C. glabrata* was evaluated on complex sugars such as soluble starch and cellulose powder. Results showed that *C. glabrata* capable of fermenting soluble starch and cellulose powder only with the aid of enzymes (amylases and cellulases). In the absence of enzymes, there was no ethanol produced. This indicated that *C. glabrata* does not produce amylolytic enzymes that breakdown starch. *C. glabrata* also unable of breaking down cellulose and requires extracellular cellulase to convert cellulose into fermentable sugar. In the last objective, *C. glabrata* was subjected to Simultaneous Saccharification and Fermentation (SSF) using total sago effluent as a biomass feedstock. Prior to the SSF, compositional analysis steps were conducted. Based on the results, total sago effluent was comprised  $97.14 \pm 0.09\%$  sago hydrolysate and  $2.86 \pm 0.09\%$

solid component (sago hampas). Further compositional analysis was carried out, and it was found that sago hydrolysate was containing  $2.23 \pm 0.86\%$  starch while sago hampas was composed of  $52.03 \pm 1.11\%$  starch,  $27.82 \pm 0.62\%$  cellulose,  $5.32 \pm 0.96\%$  hemicellulose,  $3.00 \pm 0.05\%$  lignin and  $1.95 \pm 0.03\%$  ash. SSF was conducted on total sago effluent for 5 days with the aid of enzymes (amylases and cellulases) and *C. glabrata* as a fermenting organism in a working volume of 150 ml. Results showed that the highest ethanol production was detected at 24 h, with a yield of 5.44 g/l. This indicated that *C. glabrata* could be used in bioethanol production because it can grow on lignocellulosic substrate such as total sago effluent. During the SSF, *C. glabrata* was found to be incapable of fermenting xylose and arabinose, thus resulting in low ethanol yield. Overall, *C. glabrata* has high fermentative activity for glucose, with 79.84% of theoretical yield which makes it highly suitable candidate for glucose fermentation. However, *C. glabrata* was incapable of fermenting other substrates efficiently as glucose since it only yielded low ethanol content for both sucrose and total sago effluent. Therefore, it can be concluded that *C. glabrata* is inefficient and unsuitable for industrial bioethanol processes. The efficiency and productivity of ethanol can be enhanced by performing genetic modification to increase the range of consumed sugars by *C. glabrata*.

**Keywords:** *Candida glabrata*; bioethanol; biomass feedstock; total sago effluent; fermentation; simultaneous saccharification and fermentation (SSF)

**Penilaian tentang Candida glabrata dalam Sakrafikasi dan Fermentasi Serentak (SSF)  
Jumlah Efluen Sagu**

**ABSTRAK**

Ladang sagu kebanyakannya terdapat di Sarawak dan industri sagu telah menghasilkan banyak sisa buangan setiap tahun. Sisa sagu ini berpotensi menjadi substrat bahan mentah untuk penghasilan bioetanol kerana ia boleh didapati dalam jumlah yang banyak. Dalam penghasilan bioetanol, keupayaan mikroorganisma melakukan proses fermentasi ke atas substrat adalah penting untuk mencapai hasil etanol yang tinggi. Candida glabrata biasanya terdapat dalam makanan yang ditapai seperti ragi, yang berpotensi untuk digunakan sebagai organisma fermentasi dalam penghasilan bioetanol. Oleh itu, dalam kajian ini, C. glabrata digunakan untuk proses penapaian untuk menghasilkan etanol. Objektif pertama kajian ini adalah tentang keupayaan C. glabrata dalam penghasilan etanol daripada gula mudah seperti glukosa, maltose dan sukrosa. Penapaian telah dijalankan selama 5 hari, dengan 150 rpm pada suhu ambien. Hasil kajian menunjukkan bahawa C. glabrata hanya menggunakan glukosa dan sukrosa dengan penghasilan etanol maksimum, 20.4 g/l dan 1.64 g/l. C. glabrata telah menghasilkan kandungan etanol tertinggi dalam substrat glukosa. Ini menunjukkan bahawa C. glabrata berpotensi menjadi fermentasi organisma kerana ia boleh menukar glukosa kepada etanol dengan cekap. Seterusnya, keupayaan C. glabrata dalam penapaian dinilai pada gula kompleks seperti kanji larut dan serbuk selulos. Hasil kajian menunjukkan bahawa C. glabrata boleh melakukan penapaian terhadap kanji larut dan serbuk selulos hanya dengan bantuan enzim. Tiada etanol dapat dihasilkan tanpa bantuan enzim. Ini menunjukkan bahawa C. glabrata tidak menghasilkan enzim amilolitik yang memecahkan struktur kanji larut. C. glabrata juga tidak boleh memecahkan struktur selulos dan memerlukan enzim selulase untuk menukar selulos kepada

gula. Dalam objektif akhir, C. glabrata diguna pakai untuk proses sakrafikasi dan fermentasi serentak (SSF) menggunakan jumlah efluen sagu sebagai bahan mentah. Sebelum SSF, beberapa langkah analisis telah dilaksanakan. Berdasarkan analisis, jumlah efluen sagu terdiri daripada  $97.14 \pm 0.09\%$  sisa cecair (hidrolisat sagu) dan  $2.86 \pm 0.09\%$  komponen pepejal (sagu hampas). Analisis yang lebih terperinci telah dijalankan, dan mendapati bahawa hidrolisat sagu mengandungi  $2.23 \pm 0.86\%$  kanji manakala sagu hampas mengandungi  $52.03 \pm 1.11\%$  kanji,  $27.82 \pm 0.62\%$  selulosat,  $5.32 \pm 0.96\%$  hemiselulosa,  $3.00 \pm 0.05\%$  lignin dan  $1.95 \pm 0.03\%$  abu. SSF telah dijalankan menggunakan jumlah efluen sagu selama 5 hari dengan bantuan enzim (amilase dan selulase) dan C. glabrata sebagai organisma fermentasi dalam jumlah 150 ml. Keputusan menunjukkan bahawa penghasilan etanol tertinggi dikesan pada jam ke-24, dengan hasil 5.44 g/l. C. glabrata boleh digunakan dalam penghasilan bioethanol kerana ia boleh hidup dalam substrat lignoselulosat seperti jumlah efluen sagu. Semasa SSF, C. glabrata didapati tidak dapat melakukan proses penapaian terhadap xylosa dan arabinosa, sekaligus meyebabkan penghasilan etanol yang rendah. Secara keseluruhannya, C. glabrata mempunyai aktiviti yang tinggi untuk glukosa, dengan 79.84% daripada hasil teori dan menjadikan ia berpotensi dan sangat sesuai untuk proses penapaian glukosa. Walau bagaimanapun, C. glabrata kurang efisien terhadap substrat lain kerana ia hanya menghasilkan kandungan etanol yang rendah untuk sukrosa dan jumlah efluen sagu. Kesimpulannya, C. glabrata adalah tidak cekap dan tidak sesuai untuk proses industri bioetanol. Kecekapan dan produktiviti etanol boleh dipertingkatkan dengan melaksanakan pengubahsuaian genetik untuk mempelbagai rangkaian gula yang digunakan oleh C. glabrata.

**Kata kunci:** Candida glabrata; bioetanol; bahan mentah biojisim; jumlah efluen sagu; sakrafikasi dan fermentasi serentak (SSF)

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## LIST OF ABBREVIATIONS

|                                               |                                        |
|-----------------------------------------------|----------------------------------------|
| ADF                                           | Acid detergent fibre                   |
| ADH                                           | Alcohol dehydrogenase                  |
| ANOVA                                         | One-way analysis of variance           |
| ATP                                           | Adenosine triphosphate                 |
| C <sub>2</sub> H <sub>5</sub> OH              | Ethanol                                |
| CH <sub>3</sub> CH <sub>2</sub> OH            | Ethanol                                |
| C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> | Glucose                                |
| CBD                                           | Carbohydrate binding domain            |
| CBH                                           | Cellobiodehydrase                      |
| <i>C. glabrata</i>                            | <i>Candida glabrata</i>                |
| CO <sub>2</sub>                               | Carbon dioxide                         |
| EMP                                           | Embden-Meyerhof-Parnas                 |
| EtOH                                          | Ethanol                                |
| FPU                                           | Filter paper unit                      |
| g                                             | Gram                                   |
| g/cm <sup>3</sup>                             | Gram per centrimetre                   |
| GHG                                           | Green-house gases                      |
| g/l                                           | Gram per liter                         |
| h                                             | Hour                                   |
| HPLC                                          | High performance Liquid Chromatography |
| Kg                                            | Kilogram                               |
| Kton/year                                     | Kiloton per year                       |

|                     |                                                |
|---------------------|------------------------------------------------|
| M                   | Molar                                          |
| mg/g                | Miligram per gram                              |
| MJ/dm <sup>3</sup>  | Megajoule per cubic decimetre                  |
| MJ/Kg               | Megajoule per kilogram                         |
| mm                  | Milimetre                                      |
| ml                  | Mililitre                                      |
| ml/min              | Mililitre per minute                           |
| min                 | Minutes                                        |
| NDF                 | Neutral Detergent Fibre                        |
| nm                  | Nanometer                                      |
| PDC                 | Pyruvate decarboxylase                         |
| pH                  | Potential of hydrogen                          |
| PSA                 | Phenol sulfuric acid                           |
| p-value             | Probability value                              |
| RBDC                | Rose Bengal Chloramphenicol Agar               |
| RFA                 | Renewable Fuels Association                    |
| RI                  | Revolutions per minute                         |
| RID                 | Refractive index                               |
| rpm                 | Refractive index                               |
| <i>S.cerevisiae</i> | <i>Saccharomyces cerevisiae</i>                |
| SD                  | Standard deviation                             |
| SHF                 | Separate hydrolysis and fermentation           |
| SSF                 | Simultaneous saccharification and fermentation |
| TEY                 | Theoretical ethanol yield                      |

|                    |                   |
|--------------------|-------------------|
| tons               | Tonnes            |
| $\mu\text{l}$      | Microlitre        |
| $\mu\text{m}$      | Micrometre        |
| v/v                | Volume per volume |
| w/v                | Weight per volume |
| w/w                | Weight per weight |
| YM                 | Yeast Malt        |
| $^{\circ}\text{C}$ | Degree Celsius    |

## CHAPTER 1

### GENERAL INTRODUCTION

#### 1.1 Background of the study

The demand for energy continues to rise due to population growth and developing countries (Robak & Balcerek, 2018). The continuous consumption of fossil fuel would lead to a shortage of energy resources. Furthermore, the intensive use of fossil fuels has caused greenhouse gases (GHG) emissions like carbon dioxide (Okkerse & van Bekkum, 1999; Solomon & Qin, 2013). Therefore, it is crucial to find other alternatives to sustain our energy resources. Biofuel has been introduced as one of the alternatives to substitute non-renewable energy. In fact, biofuels have been used for years with the purpose to increase energy self-sufficiency, minimize import costs and strengthen domestic agricultural development (Kovarík, 2013; Araújo, 2017). Bioethanol ( $\text{CH}_3\text{CH}_2\text{OH}$ ) from biomass is one of the most widely used biofuels across the world. Bioethanol has been used as prime renewable biofuel for transportation sector in some countries such as Brazil, US other European countries (Hahn-Hägerdal et al., 2006).

According to Vohra et al. (2014), bioethanol is a liquid biofuel produced from biomass feedstocks and conversion technologies. The important key-point in the production of bioethanol from biomass is the conversion of the residual materials (biomass) into advantageous products like fermentable sugars (Mezule et al., 2015) and bioethanol derived from sugar fermentation (Shahsavarani et al., 2013). Biomass refers to any organic matter that comes from biogenic sources and is available on a renewable basis (Ahorsu et al., 2018). In bioethanol production, the suitable biomass should allow the growth of the microorganism

without interfering the product recovery. Plus, the biomass must be economically viable and available abundantly for the industrialization of the fermentation process (Oda & Nakamura, 2009; Srichuwong et al., 2009; Behera et al., 2010). Biomass contains high content of lignocellulosic components, which comprises of cellulose, hemicellulose and lignin. These long-chain polysaccharides can be broken down to yield a mixture of sugars which can be further converted to ethanol (Goh et al., 2010). Biomass is mostly derived from non-food crops or waste materials such as wood, straw, grass, corn stalks and sago wastes.

In this study, total sago effluent is chosen as biomass substrate. Total sago effluent is total wastes or residues from sago industries that usually channelled into the nearby river. According to Phang et al. (2000), about 10 - 22 tons of wastewater are discharged into the rivers per day from factories. These wastes need to be handled properly to avoid water pollution. Total sago effluent consists of sago hydrolysate, which is the liquid part while solid component called as sago hampas. Total sago effluent has been reported to contain a high amount of lignocellulosic materials which can be found in sago hampas (Awg-Adeni et al., 2012). As mentioned previously, biomass with high lignocellulosic components is suitable for bioethanol production. The hydrolysate also contributes to the production of bioethanol as the hydrolysate contains starch that can be converted into fermentable sugars as well (Ritslaid et al., 2010). Singhal et al. (2008) also stated that sago effluent has a relatively very high carbon to nitrogen ratio (105:0.12), which makes it a suitable substrate for fermentation and the production of high protein fungal biomass.

Production of bioethanol from biomass such as total sago effluent requires several steps, starts from biomass pretreatment and hydrolysis, followed by hydrolysate fermentation and ethanol separation (Limayem & Ricke, 2012). At present, many applications are available for the conversion of lignocellulosic to fuel ethanol, where the only difference lies on the catalyst used for the breaking down of polysaccharides in the raw biomass (Kádár et al., 2004). For example, an enzyme can be applied in pretreatment or enzyme hydrolysis to break down the hemicellulose fraction using acids or bases. It will break down the large sugar molecules in the substrate into smaller molecules so that it will be easier to be fermented into ethanol. Traditionally, enzyme hydrolysis step is carried out separately from fermentation where the enzyme hydrolysis is conducted first before the fermentation step. However, there is another way to conduct the enzyme hydrolysis in which both enzyme hydrolysis and fermentation are carried out at the same time in the same vessel (Gauss et al., 1976). This idea is called SSF or Simultaneous Saccharification and Fermentation.

SSF was introduced by Gauss et al. (1976), in which the author reported that production of glucose in separate enzymatic hydrolysis was low because of the end-product inhibition, that resulted from the process of hydrolysis by glucose and cellobiose. The author then managed to obtain higher ethanol yield when employing the idea of SSF. Takagi et al. (1977) defined SSF as a process that combines enzymatic hydrolysis of cellulose or starch with the simultaneous fermentation of its derived sugars to ethanol. SSF is preferable in some research because of its advantages. Since the process of enzymatic hydrolysis of cellulose or starch is combined with the fermentation, the whole cost and time can be cut and shorten (Öhgren et al., 2006). This could increase efficiency of ethanol production during fermentation.

Efficiency of ethanol productions also influenced by species of microorganism used. The desired microorganism should be able to convert sugars in substrates to ethanol effectively. The most common examples are *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Fusariumoxys porum* (Chaudhary & Chincholkar, 1986). Among them, *S. cerevisiae* has been used since a long year ago in alcoholic fermentation due to their characteristics in ethanol production. Another species that has similar characteristics to *S. cerevisiae* is *Candida glabrata* (Kurtzman & Robnett, 1998; Merico et al., 2007). This species can be found in fermented foods, such as *ragi* (Kofli & Davaon, 2010). *C. glabrata* was reported to yield higher ethanol content and able to grow under anaerobic conditions (Merico et al., 2007). Watanabe et al. (2008) also stated that *C. glabrata* is a thermotolerant yeast, which should be considered as a potential strain for bioethanol production, especially for SSF. Thus, the main objective of this study is to investigate the abilities of *C. glabrata* in producing ethanol from a variety of substrate especially, biomass such as total sago effluent.

## **1.2 Problem statement**

Improper disposal of sago effluent from the sago industries can lead to water pollution, which will greatly affect the natural ecosystem. Due to this problem, many studies have been conducted to find alternative means to mediate this problem. Previous studies have reported that sago effluent contains high percentages of lignocellulosic materials which can be converted into bioethanol. Therefore, its utilization of bioethanol production not only benefits our ecosystem as a result of volume reduction, but it can also solve the problem regarding fossil fuel usage. Moreover, the demand for ethanol is rising, and this alternative could be one way to supplement this need.

### **1.3 Objective of the Study**

The main objective of this study was to study the ability of *C. glabrata* in Simultaneous Saccharification and Fermentation (SSF) of total sago effluent. Therefore, the whole study is divided into three parts according to these specified objectives:

- i. To evaluate the ability of *C. glabrata* in producing ethanol from simple sugars such as glucose, sucrose and maltose
- ii. To investigate the ability of *C. glabrata* in producing ethanol from complex sugars such as starch and cellulose
- iii. To evaluate the production of bioethanol from Simultaneous Saccharification and Fermentation (SSF) of total sago effluent

### **1.4 Organization of the Thesis**

The thesis is divided into six chapters. The first chapter is Introduction Chapter. This chapter provides a brief introduction on overview of the background, problem statement and objectives of the study. Chapter 2 is literature review of related topics and findings from other previous research in the same field. This includes an introduction to biofuel bioethanol, the detail process of production of bioethanol such as substrates, microorganism involved and other conditions crucial during the production of bioethanol.

Chapter 3 is materials and methodology in which all the materials used in this study are listed. In this chapter, the methods that are employed throughout this study are stated in detail. Basically, this chapter shows a flowchart of all lab works, which includes objective

1, objective 2 and objective 3. Chapter 4 presents the results that are collected throughout this study. The results are including evaluation of cell growth, HPLC profiling and total carbohydrate analysis using PSA method. The results are tabulated in graph and the statistical analysis of data is done by using SPSS.

Chapter 5 is discussion chapter. This chapter provides detail justification based on the results obtained in the previous chapter. The next chapter is Chapter 6, which is the final chapter of the study. This chapter summarizes the whole study with a general conclusion and some recommendations for future work are discussed in this chapter.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Biofuel

Excessive dependant on fossil fuels has undoubtedly led to the diminishing of non-renewable supply. It was reported that the demand for energy has expanded because of transportation, heating and mechanical handling (Hahn-Hägerdal et al., 2006). Aditiya et al. (2016) also mentioned that the remarkable rise in petroleum consumption mostly caused by the transportation sector. As shown in Figure 2.1, the usage of petroleum derivatives such as oil, coal and flammable gas greatly surpasses the entire utilization of sustainable power source and hydroelectricity. The continuous consumption of fossil fuel causes depletion of natural resources and thus leads to other issues like global warming (Muktham et al., 2016).

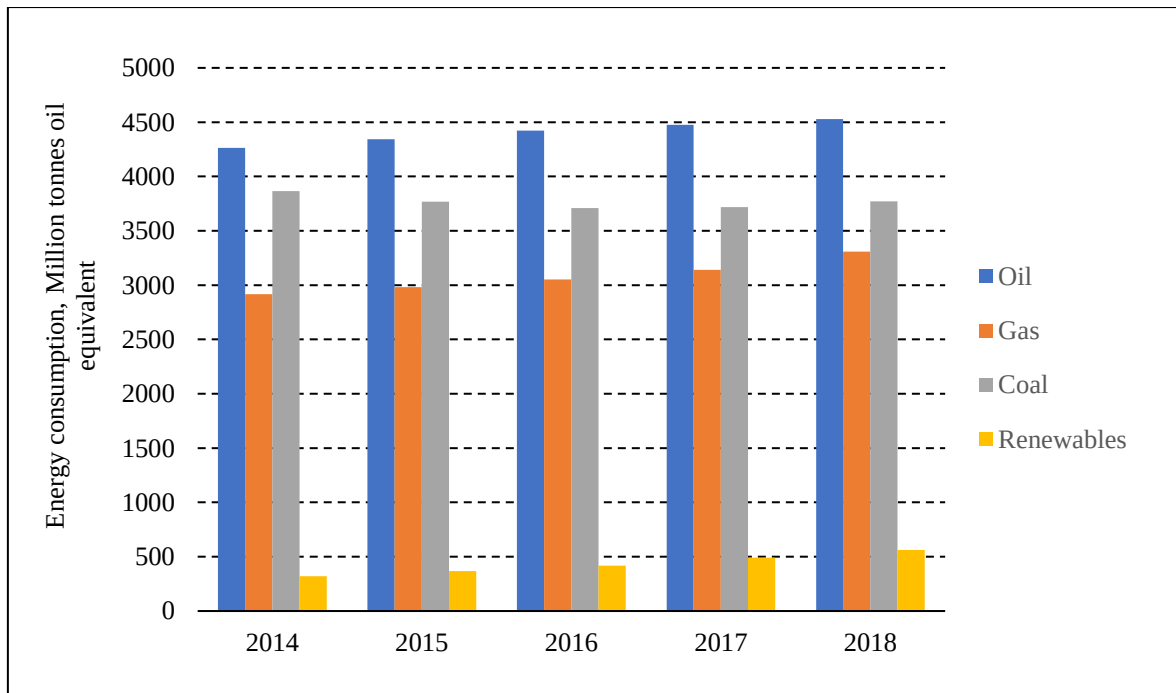


**Figure 2.1:** World energy consumption by fuel type (Adapted from Muktham et al., 2016) (Data collected from British Petroleum, 2019).

Global warming is one of the major problems caused by excessive consumption of fossil fuel, due to the GHG emission or so-called greenhouse gas emission in the atmosphere. Ballesteros et al. (2006) reported that the level of GHG in the earth's atmosphere has risen dramatically in the past years. Energy crisis and global warming have become the major concerns for developed economies and emerging countries. Therefore, the discovery of renewable energy is significant to handle this issue. The desired renewable energy supply must comply with a significant concurrent decrease in GHG emissions and able to avoid the risk of an energy shortage worldwide (Farrell et al., 2006; Mansouri et al., 2016). Bioenergy such as biofuel is deemed as one of the suitable alternatives to replace fossil fuels. Other examples of biofuels are biodiesel, biomethanol, biogas, bio-syngas and biohydrogen (Demirbas, 2008).

Biofuels are solid, liquid or gaseous fuels produced from renewable energy sources. Among these, liquid biofuels are the most significant as they can be used to substitute petroleum fuels (Tye et al., 2016). Biofuels are known as non-polluting and can be obtained from renewable energy sources such as biomass whereas petroleum is a polluting fuel and acquired from non-renewable energy sources (Demirbas et al., 2009). At present, biomass is considered as a potential energy source as it can lead to sustainable development (Monique et al., 2003).

## **2.2 Classification of biofuels**

In general, biofuels can be classified into three classes of generations depending on the feedstocks used (Nigam & Singh, 2011). First-generation of biofuel is mainly derived from

sugars or starchy materials while second-generation of biofuel comes from lignocellulosic raw materials. Lin and Tanaka (2006) stated that lignocellulose is the most abundant source of biomass and these raw materials are reported as largely unutilized. Table 2.1 represents different generations of biofuels with their characteristics.

**Table 2.1:** Different generation of biofuels (Adapted from Sikarwar et al., 2017).

| <b>First-generation</b>                                                                                                                                                                                                               | <b>Second-generation</b>                                                                                                                                                                                                                  | <b>Third-generation</b>                                                                                                                                                                                                                                                                                                             |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"> <li>- Derived from food crop</li> <li>- Biochemical methods like fermentation or hydrolysis are employed to convert them to biofuels</li> <li>- Responsible for food versus fuel debate</li> </ul> | <ul style="list-style-type: none"> <li>- Produced from non-food crops (lignocellulosic biomass)</li> <li>- Biochemical and thermochemical are used to synthesize biofuels</li> <li>- “Biomass to liquid” fuel concept employed</li> </ul> | <ul style="list-style-type: none"> <li>- Derived from algae &amp; other microbes.</li> <li>- Cultivable land not required</li> <li>- Fastest growing feedstocks among all other sources</li> <li>- Biochemical and thermochemical are employed</li> <li>- Extensive downstream processing such as dewatering is required</li> </ul> |

### 2.2.1 First-generation of biofuel

Sugars like sugarcane can be converted directly into ethanol. In comparison to starchy and lignocellulosic materials, the conversion process is simpler as the yeast cells can instantly break down this disaccharide sugar (Cardona & Sanchez, 2007). Rossillo-Calle and Walter (2006) stated that approximately 95% of the ethanol production in the world is obtained from agricultural. The production of bioethanol from sugar crops accounts for 40% of bioethanol manufacturing and almost 60% of sugar crops. Bioethanol derived from sugar or starchy materials is categorized under conventional technology (Chin & H’ng, 2013). Several industrial ethanol productions have been reported producing ethanol from various starchy materials such as corn, wheat, starch and potatoes, cassava root (Lindeman &

Rocchiccioli, 1979), corn stover (Wilke et al., 1981; Kadam & McMillan, 2003), and starch (Maisch et al., 1979). However, for starchy materials, the hydrolysis process must be conducted first before the fermentation process.

Like starchy materials, lignocellulosic materials also need to undergo hydrolysis process prior to the fermentation process. Lignocelluloses are mainly derived from agricultural and forestry residues, along with waste materials, such as wood and agricultural residues. Lignocelluloses contain cellulose which can be converted into fermentable sugars with the aid of enzymes. Then, these fermentable sugars can be fermented by yeasts to produce ethanol (Lin & Tanaka, 2006). Bioethanol from lignocellulosic materials is described as advance biofuel technologies (Chin & H'ng, 2013) or so-called as second-generation bioethanol.

### **2.2.2 Second-generation biofuel**

Second-generation bioethanol has received extensive attention as it offers a great deal to substitute fossil fuels without interfering in food supplies (Goh et al., 2009). According to Thompson and Meyer (2013), the second generation of biofuel including bioethanol is based on non-food raw material and thus does not compete against food supplies. Those are from non-edible sources, in which the main components are cellulose, hemicellulose and lignin (Sun & Cheng, 2002). Furthermore, lignocellulosic materials are considered a better option of potential feedstock as these substrates can be found in an abundant amount.

To date, lignocellulosic biomass is regarded as the cheapest substrate for bioethanol production. More than 90% of global production of plant biomass is lignocellulosic biomass, approximately about  $200 \times 10^9$  tons per year and it is estimated that about  $8\text{--}20 \times 10^9$  tons of the primary biomass remain potentially accessible (Lin & Tanaka, 2006). According to Ratnam et al. (2003), good production of bioethanol relies on the availability to produce a high amount of ethanol concentrations and at the same time maintaining high-quality yield at the end of the process.

### **2.3 Emergence of bioethanol in the world and Malaysia**

Bioethanol also known as ethyl alcohol ( $\text{CH}_3\text{CH}_2\text{OH}$ ) is an end-product from a process called fermentation of sugars using microorganisms like yeasts and bacteria (Chin & H'ng, 2013). According to Demirbas et al. (2009), the first prototype of internal combustion engines was developed by Samuel Morey in 1826 and ethanol was started to be used as fuel in 1876. Rosegrant et al. (2006) in his report stated that the “Aggressive Biofuel Growth” scenario aims to substitute 10% of gasoline production with biofuels by 2010, 15% by 2015, and 20% by 2020. Bioethanol is not a new approach since it has been used since the early 1900s in Europe and the United States. However, it was disregarded because of its high production cost contrasted with petrol. Despite that, bioethanol industry was continued to emerge due to the oil price crisis in the 1970s (Balat, 2009). According to Badger (2002), nowadays, countries that experience oil price crisis are producing ethanol from cellulosic substrates.

For global ethanol market, United States is known as the world's largest producer of ethanol, with 58% of global output in 2014, while Brazil is in second place, with 25% and followed by European Union with 6% and the remaining 11% is produced by the rest of world, as shown in Figure 2.2 (RFA, 2015).

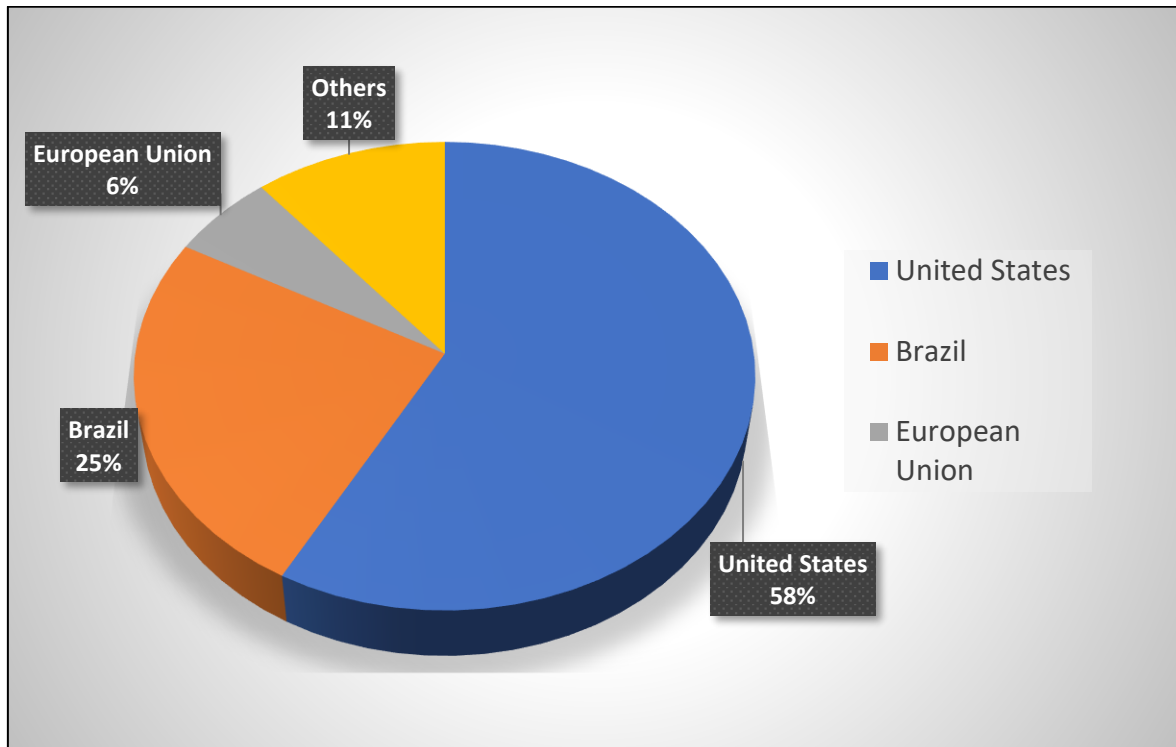


**Figure 2.2:** Global ethanol production in 2014 (RFA, 2015).

According to Solomon et al. (2007), over 95% of ethanol production in the United States is derived from corn, while the rest are derived from wheat, barley, cheese whey, and beverage residues. In Brazil, the main substrates used are derived from sugar cane and molasses. Other countries also involved in the production of bioethanol from traditional food crops such as wheat (France, England, Germany and Spain), cassava (Thailand, Nigeria) and sorghum (India). The feedstocks used depend on location and dominant agricultural products (Mojovic et al., 2006).

### **2.3.1 Consumption of bioethanol in Malaysia**

Malaysia is no exception, which also experiences the issue of sustainability due to the demand of energy consumption. Transportation (40.3%) and industrial (38.6%) are the leading sectors utilizing in Malaysia. Meanwhile, the remaining energy is supplied to other main sectors such as residential, commercial and agricultural and others (Tye et al., 2011). Therefore, energy demand in Malaysia is expected to grow higher in rate. Global warming is one of the critical problems which arises to follow the energy crisis. More than 19% of the total carbon dioxide emission in Malaysia is caused by the transportation sector. Despite the small population, an average emission of 7.5 tons of carbon dioxide per capita has been reported in Malaysia. In conjunction with this, The National Biomass Strategy 2020 proposes a mandate of bioethanol blending of 10% in petrol fuel by 2020 to reduce the GHG emissions (Chin & H'ng, 2013).

Malaysia is one of the countries that joined collectively to reduce the consumption of non-renewable energy to ensure the development of economy growth in Malaysia and at the same time capable to meet the commitment of UN convention on Climate Change and Kyoto Protocol (Mohamed & Lee, 2006). In 2005, the National Biofuel Policy was launched and became the main influence for the production and consumption of biofuel in Malaysia. Owing to the issues of market competency, energy crisis and climate change, bioethanol has been introduced in the recent policy of diversification of bioenergy.

According to Tan et al. (2008), the focus on renewable liquid biofuels is mainly limited to bioethanol and biodiesel regardless of numerous studies that have been conducted in the past. Currently, it has been reported that very little production of bioethanol from biomass

in Malaysia although there is a great potential for the use of oil palm biomass (Kennedy & Ahamad, 2007). This is because the idea of bioethanol from lignocellulose materials is still considered as a new approach in Malaysia. On top of that, the development of lignocellulose-related technologies is also not very well-established in the world (Goh et al., 2010). On the other hand, Malaysian market for bioethanol is said to be more potentially larger than biodiesel, mainly due to the considerably more significant proportion of the vehicle fleet runs on gasoline (Tye et al., 2011).

## **2.4 Characteristics and advantages of bioethanol**

Bioethanol is described as a colourless liquid (Chin & H'ng, 2013). Bioethanol is considered as a carbon 'neutral'. It is essentially free from sulfur which is harmful to living organisms. Besides, the total burning of bioethanol merely delivers carbon dioxide and water (Goh et al., 2010). Therefore, ethanol can be considered as a great alternative to substitute oil as it is clean and renewable combustible (Almeida & Silva, 2006; Bai et al., 2008). This is supported by Krylova et al. (2008), where the author reported that the combustion of ethanol is much cleaner than petroleum fuel. It mainly because it contains oxygen and as a result, the emission of toxic substances is lower. Aside from that, bioethanol that used as a transportation fuel able to aid in reducing carbon dioxide buildup in two significant ways. One of them is by displacing the use of fossil fuels. Another way is by recycling the released carbon dioxide when it is combusted as fuel. When using bioethanol instead of fossil fuels, the emissions from fossil fuel can be avoided, and the content of carbon dioxide in fossil fuels can remain in storage. Besides, burning ethanol instead of gasoline can limit carbon emissions through more than 80% and at the same time able to eliminate the release of acid-

rain-causing sulfur dioxide (Schwartz, 1989). Table 2.2 summarizes the physiochemical properties of ethanol.

**Table 2.2:** Physiochemical properties of ethanol (Adapted from Krylova et al., 2008).

| Characteristics                                                         | Value                              |
|-------------------------------------------------------------------------|------------------------------------|
| <b>Physical properties</b>                                              |                                    |
| Density, g/cm <sup>3</sup>                                              | 0.79                               |
| Saturated vapour pressure at 38 °C, Torr                                | 50                                 |
| Boiling temperature, °C                                                 | 78.5                               |
| Permittivity, $\epsilon$                                                | 24.3                               |
| Water solubility, g/l                                                   | $\infty$                           |
| <b>Chemical properties</b>                                              |                                    |
| Formula                                                                 | CH <sub>3</sub> CH <sub>2</sub> OH |
| Molecular weight                                                        | 46.1                               |
| Elemental composition, %                                                |                                    |
| - Carbon                                                                | 52.1                               |
| - Hydrogen                                                              | 13.2                               |
| - Oxygen                                                                | 34.7                               |
| <b>Thermal properties</b>                                               |                                    |
| Net calorific value, MJ/kg (kcal/kg)                                    | 26.82 (6400)                       |
| Ignition temperature, °C                                                | 35                                 |
| Specific heat capacity, MJ/kg, (kcal kg <sup>-1</sup> K <sup>-1</sup> ) | 0.03 (0.60)                        |
| Melting temperature, °C                                                 | -115                               |

Ethanol also can be blended or mixed with gasoline engines, as it has a higher-octane number and higher heat of vaporization (Hahn-Hägerdal et al., 2006). According to Sebayang et al. (2016), the primary standards of the bioethanol to substitute, or to be blended with, petrol is prescribed officially under ASTM D4806 standard, which sets the quality requirements of bioethanol for the spark-ignited engine. In short, ethanol fuel that used as energy replacement is essentially considered as feasible, economical, efficient, safer, eco-friendly, and a renewable alternative to conventionally used fossil fuel (Prasad et al., 2007; Zabed et al., 2014). Aside from the transportation sector and renewable energy production, bioethanol also intensively being used in other fields such as pharmaceuticals, cosmetics, beverages,

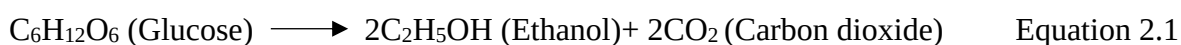
industrials and medical sectors as well. For example, bioethanol could be used to supply the existing chemicals industry (Chin & H'ng, 2013). Table 2.3 summarizes the main advantages of bioethanol.

**Table 2.3:** Bioethanol fuel properties (Adapted from Deenanath et al., 2013).

| Bioethanol fuel property                                                   | Advantages                                                                                                                                          | References                                                           |
|----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|
| High oxygen content (35% w/w)                                              | - Increased combustion efficiency<br>- Reduced hydrocarbon and carbon monoxide emissions                                                            | De Menezes et al., 2006<br>Demirbas, 2008<br>Sanchez & Cardona, 2008 |
| High octane number (107) and high latent heat of vaporization (0.91 MJ/kg) | - Prevents premature ignition and cylinder knocking<br>- Spontaneous ignition in internal combustion engines when bioethanol petrol blends are used | Demirbas, 2008<br>Turner et al., 2011<br>Walker, 2011                |
| Low energy content (21.2 MJ/dm <sup>3</sup> )                              | - Increased compression ratio<br>- Decreased burn time<br>- Increased power                                                                         | Demirbas, 2008<br>Jegganathan et al., 2009<br>Walker, 2011           |

## 2.5 Biological process of ethanol production

Ethanol is an end-product from a process called fermentation. Fermentation process can be defined as a biological process in which fermentable sugars are converted into cellular energy and thus producing end-products called ethanol and carbon dioxide in the absence of oxygen (anaerobic process) (Chin & H'ng, 2013). Pyruvate can be fermented to ethanol by sequential reactions of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) while losing one carbon as carbon dioxide (Kang & Lee, 2015). Theoretically, each kg of glucose and xylose can produce 0.49 kg carbon dioxide with 0.51 kg of ethanol (Hamelinck, 2005). The complete equation is shown in Equation 2.1.



One of the vital key factors in the regulation of fermentation in yeast is oxygen and yeasts can be classified based on the role of oxygen in their metabolism. According to Merico et al. (2007), three primary classifications of yeasts are obligatory aerobic, in which only respiratory metabolism, followed by facultative fermentative or respiro-fermentative, which displaying both respiratory and fermentative metabolism and lastly obligatory fermentative. In addition, Demirbas (2004) also mentioned that the other additional factors that play a significant role in the fermentation process are the microbes' growth rate and genetic stability such as tolerance of inhibitors, osmosis and alcohol itself such as productivity and ethanol yield.

In industrial fermentation, there are two regulatory effects that important known as the Pasteur and the Crabtree effects. van Dijken and Scheffers (1986) stated these effects are examples of special competition between respiration and fermentation of glucose. The Pasteur effect can be described as activation of anaerobic glycolysis in order to meet cellular ATP demands owing to the lower efficiency of ATP production by fermentation compared with respiration. Meanwhile, the Crabtree effect refers to the occurrence of alcoholic fermentation under aerobic conditions. in industrial fermentation (Rodrigues et al., 2006; Hagman et al., 2014). Conventional yeasts such as *S. cerevisiae* utilizes glucose by fermentative pathway (Crabtree positive) and while some nonconventional yeasts are predominantly oxidative (Crabtree negative). However, noted that among non-conventional yeasts, there are also Crabtree-positive ones.

## **2.6 Bioethanol production from lignocellulosic biomass**

Lignocellulose is said to be one of the renewable resources and abundant biopolymers in the earth (Wang et al., 2008). It has been recognized that the production of bioethanol from renewable biomass gained considerable attention due to its abundant availability (Patel et al., 2007). As indicated by Sánchez and Cardona (2008), lignocellulose is the most plentiful renewable biomass produced from photosynthesis, and its annual production was estimated in  $1 \times 10^{10}$  MT worldwide. For the industrial purpose or in large scale production of cheap commodities such as fuels, all the chemical used, and the raw material selected must be very cheap (Miller & Churchill, 1986; Dahod, 1999). The expanded interest for crude oil, manifested in trading prices approaching \$80/barrel in 2006, has renewed the interest in exploiting lignocellulosic biomass not only for liquid transportation fuel but also for the manufacturing of chemicals and materials such as carbohydrate-based biorefineries (Ohara, 2003; Kamm & Kamm, 2004; Farrell et al., 2006; Gray et al., 2006; Herrera, 2006).

As mentioned in the past, lignocellulosic biomass is said to be more difficult to be converted into bioethanol unlike other substrates as it has a complex structure (Liu & Shen, 2008). A few major steps are required for the biological process of converting the lignocellulose to fuel ethanol. Firstly, the process started with step known as delignification, which is to liberate cellulose and hemicellulose from their complex with lignin, then followed by depolymerization of the carbohydrate polymers to produce free sugars, and finally will be a fermentation of mixed hexose and pentose sugars to produce ethanol (Lin & Tanaka, 2006).

Lignocellulosic and starchy materials in the biomass are convertible to fermentable sugars that can be further processed, which results in the production of bioethanol as the product. In other words, the components like cellulose, or any carbohydrates, that can be found in biomass, can be converted into bioethanol through hydrolysis, fermentation, and distillation. The main product of cellulose hydrolysis is glucose and other six-carbon sugars, while hemicellulose hydrolysates are typically xylose and other five-carbon sugars (Brienzo et al., 2009; Kamoldeen et al., 2017). Goh et al. (2010) stated that the efficiency of the conversion process is often influenced by the type of lignocellulosic. For instance, if the biomass comprises of a larger ratio of cellulose, the conversion is predicted to be higher than those with a lower ratio. According to Demirbas (2005), the type of source of sugar also determines the quality of ethanol produced from fermentation, whereas the author mentioned that glucose can be fermented into ethanol with an efficiency of about 75%, while 50% with xylose.

According to Goh et al. (2010), equipment and process operation requires additional cost as lignocellulosic materials are unintended to be readily accessible as a carbon source. As a result, high capital and operation cost becomes the main challenge for the whole process. For example, Chen and Qiu (2010) stated that the costs of cellulase and ethanol distillation using lignocellulose as raw material, accounts for 30% to 50% and 20% of the total cost, respectively, as compared to using grain, where the corresponding expenses are 8% and 14% of the total cost. However, Mussatto et al. (2010) noted that vast majority of the lignocellulosic materials are byproducts of agricultural activities and industrial residues and it was believed that lignocellulosic materials able to offer great potential to produce fuel

ethanol at large scale and a global consumption as a renewable fuel and thus it would make lignocellulosic biomass as the main feedstock for production of bioethanol in future.

Over the past few decades, the agriculture industry has significantly contributed to the economy. Hence, there is a substantial amount of lignocellulosic agriculture wastes produced every year. As it produces a huge amount of agriculture waste, biomass has turned into the most important sustainable source in Malaysia (Lau et al., 2009; Goh et al., 2010). Therefore, the production of bioethanol from lignocellulosic agriculture waste is introduced to provide another alternative way of converting these wastes into valuable products (Goh et al., 2010). On the other hand, these wastes could cause another problem such as pollution if not get treated properly. As mentioned by Got et al. (2010), the utilization of these wastes could solve the disposal problem. Moreover, this could reduce the cost of waste treatment throughout the whole industrial process.

## **2.7 Lignocellulosic components**

Lignocellulosic biomass is derived from animals, plants, trees, grasses, crops, vegetables, agroforest residues, and aquatic plants. Plants fix carbon dioxide through the process called photosynthesis, where it will synthesize compounds. The compounds are primary and secondary metabolites. The primary metabolites are including carbohydrates such as sugars, cellulose, hemicellulose, starch and lignin (Soni et al., 2018). Figure 2.3 shows the main components of lignocellulosic materials in biomass. All types of lignocellulosic raw materials contain cellulose and hemicellulose polymers built up by long chains of sugar monomers bound together by lignin. A previous study mentioned that lignocellulosic

biomass contains 35-40% (w/w) cellulose, 25-40% (w/w) hemicellulose and 10-20% (w/w) lignin, on the daily basis of dry biomass (Amin et al., 2017).

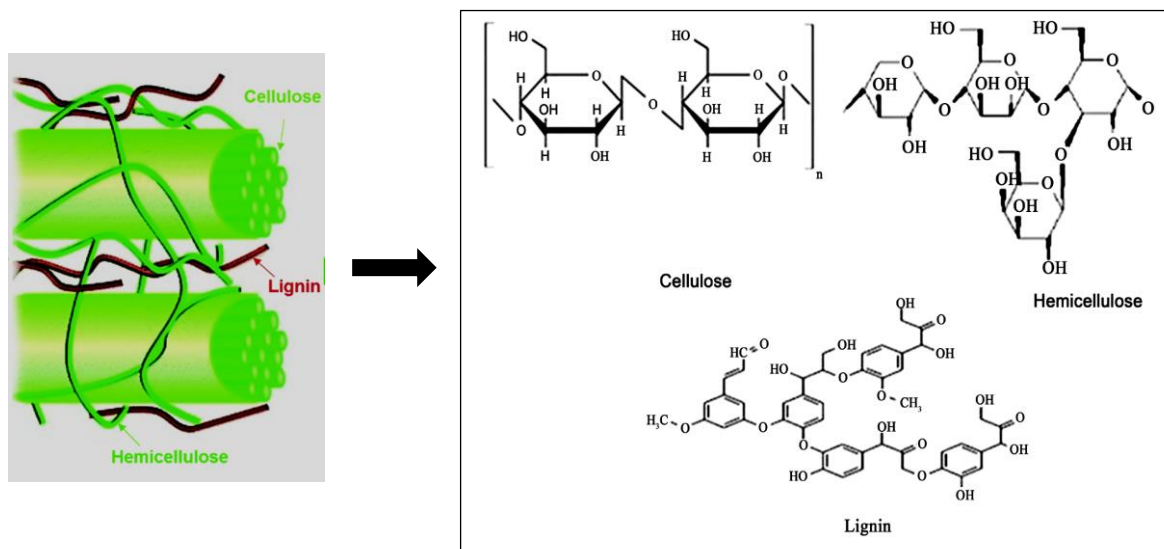


**Figure 2.3:** Lignocellulosic biomass structural components (cellulose, hemicellulose and lignin) (Adapted from Muktham et al., 2016; Zhang et al., 2016).

### 2.7.1 Cellulose

Cellulose is composed of repeating units of cellobiose or can be described as a polysaccharide that consists of a linear chain of D-glucose monomers (100-20,000) linked together by  $\beta$ -1, 4 glycosidic bonds (Zhang & Lynd, 2004). The 3D structure of cellulose consists of sheets of glucopyranose rings lying in a plane with sheets stacked on one another to form a highly organized form (Soni et al., 2018). The cellulosic network has a complex architecture composed of microfibrils arranged in a bundle, in which the microfibril is composed of 36-1200 cellulose chains (Zhang & Lynd, 2004; Gupta & Vurma, 2015). Each microfibril is about 10 to 20 nm in diameter. Figure 2.4 shows the structure of cellulose and arrangement of microfibril.

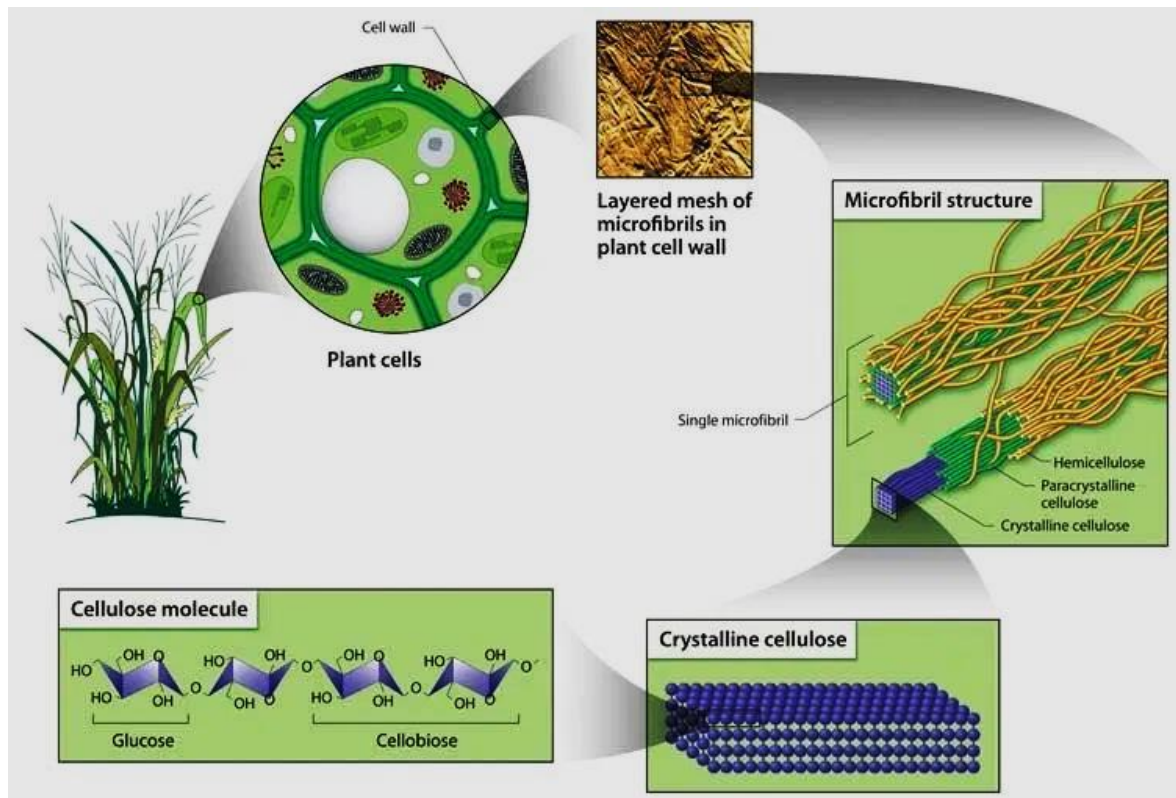


**Figure 2.4:** Arrangement of macro and microfibrils in cellulose structure (US DOE, 2005).

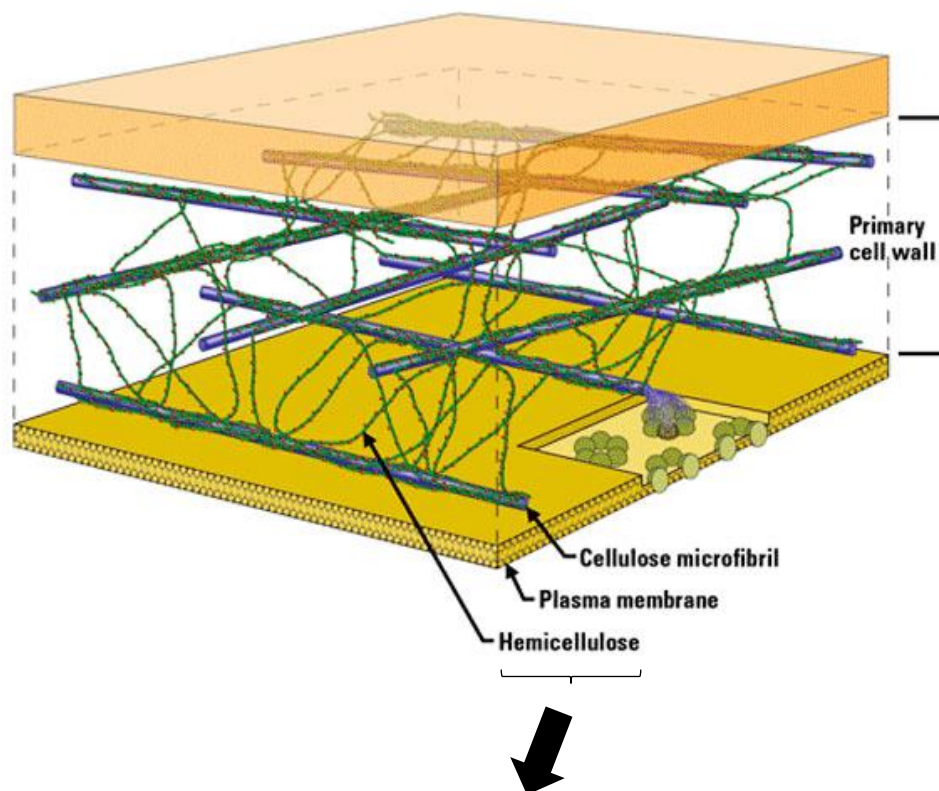
In nature, cellulose has a certain degree of crystallinity that is formed by hydrogen bonding along the chains (Li et al., 2010). Therefore, it needs a high temperature and pressure to break the rigid crystalline structure (Deguchi et al., 2006). The rigidity of the cellulose microfibril is mainly strengthened within a matrix of hemicelluloses and pectins. Pectins are non-cellulosic acidic cell wall polysaccharides, where it can be divided into three classes known as homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II. (Wyman et al., 2005; Harhold et al., 2010). The main functions of pectins in the plant cell wall are like a sol-like matrix. It provides water and ion retention, and at the same time also supports and facilitates the cell wall modifying enzymes. It aids in cell wall porosity, cell-to-cell adhesion, cell expansion, cell signalling, developmental regulation, and defense mechanism.

It also promotes pollen tube growth, seed hydration, leaf abscission, and fruit development (Wyman et al., 2005; Mohnen, 2008).

Generally, cellulose consists of two different forms, which are Ia and Ib. The first form, Ia is represented primarily in bacterial and algal cellulose, while the second form, Ib is the dominant form present in higher plants (Lima et al., 2014). Yang et al. (2011) mentioned that the presence of cellulose crystallinity is believed to affect hydrolysis process, as such slower hydrolysis rate and lower enzyme adsorption are taking place when crystallinity is high.

### **2.7.2 Hemicellulose**

Hemicelluloses are branched biopolymer that consists of pentoses, hexoses and uronic acids (Girio et al., 2010). Hemicelluloses are known as non-cellulose, non-pectin cell wall heteropolysaccharides (Wyman et al., 2005). The word “hemicellulose” is made up of two words Hemi and Cellulose, in which hemi means peripheral. Hence, the whole meaning of hemicelluloses is peripheral cellulose (Chandra et al., 2015). It can also be described as a polysaccharide which made up of chains xylose where the side chains contain arabinose, galactose, mannose, glucose, acetyl and other sugar groups (Figure 2.7). According to Chandra et al. (2015), there are several types of hemicelluloses such as xyloglucan, xylans, mannans and glucomannans, and  $\beta$ -(1  $\rightarrow$  3, 1  $\rightarrow$  4)-glucans. Xylan is known as the predominant hemicellulose in most plant cell walls, where it mainly comprising about 1/3 of the total plant biomass. Figure 2.5 shows the structure of hemicellulose and the main sugars found in hemicellulose.



|           |            |              |             |              |
|-----------|------------|--------------|-------------|--------------|
|           |            |              |             |              |
| a) xylose | b) mannose | c) galactose | d) rhamnose | e) arabinose |

**Figure 2.5:** Hemicellulose structure and its sugar groups (Adapted from US DOE, 2005; Dutton, n.d.).

According to Bastawde (1992), hemicellulose is the second most abundant polysaccharide available in nature after cellulose. Unlike cellulose, hemicelluloses are typically easier to hydrolyze due to its more amorphous property (Aditiya et al., 2016). Generally, hemicelluloses are classified according to the main sugar residue in the backbone like xylans,

mannans and glucans. However, xylans and mannans are the most prevalent (Wyman et al., 2005). As reported, xylans became five to sevenfold more digestible which could improve significantly the accessibility of cellulose to enzymatic hydrolysis (Grohmann et al., 1989).

### **2.7.3 Lignin**

The last component is lignin, which is a complex hydrophobic, cross-linked aromatic polymer composed of three types of substituted phenols (phenylpropanoids) (Hendriks & Zeeman, 2009; Menon & Rao, 2012). Lu et al. (2002) mentioned that lignin is believed to resist cellulases enzymatic activities, which results in lower hydrolysis quality. Therefore, the presence of lignin in hydrolysis is unfavorable, and lignin should be removed to maximize the effectiveness of hydrolysis. In lignin removal, fibrils of microcellulose are openly exposed and neatly separated, thus providing better access for the cellulases (Yang & Wyman, 2006; Jeoh et al., 2007). This also supported by Yuldashev et al. (1993), where the author reported that many studies have been performed in the effort of delignification of the materials to yield better fermentable sugars. For example, a study on the effect of milling pre-treatment in cotton stalks reported that pre-treated cotton stalks produce lower lignin (0.6%) and higher cellulose (92%) compared to the one without milling pre-treatment (26.4% of lignin and 44% of cellulose) (Yuldashev et al., 1993).

## 2.8 SSF process and Enzymes application

The crucial steps for production of bioethanol from lignocellulosic materials are started from physicochemical pretreatment (Alvira et al., 2010; Uppugundla et al., 2014), hydrolysis or saccharification (Kumar & Murthy, 2013), fermentation, and distillation or end-products recovery (Balat et al., 2008; Phillips et al., 2013). The whole steps are summarized in Figure 2.6. Among all these steps, the saccharification step is most important for the maximum production of bioethanol. Saccharification refers to the hydrolysis reaction that occurred before fermentation. The hydrolysis process aims is to separate long chain of carbohydrate (from cellulose or starch) with the addition of water molecule and is usually catalyzed or aided by enzyme or acid. Hydrolysis process is essential since the microorganism can only digest a simpler sugar form obtained from the complex carbohydrate of biomass. However, enzymatic hydrolysis is known for its economical challenge due to the high cost of enzymes. Furthermore, enzymatic hydrolysis is considered impractical for commercial purposes (Mosier et al., 2002; Ferreira et al., 2009).

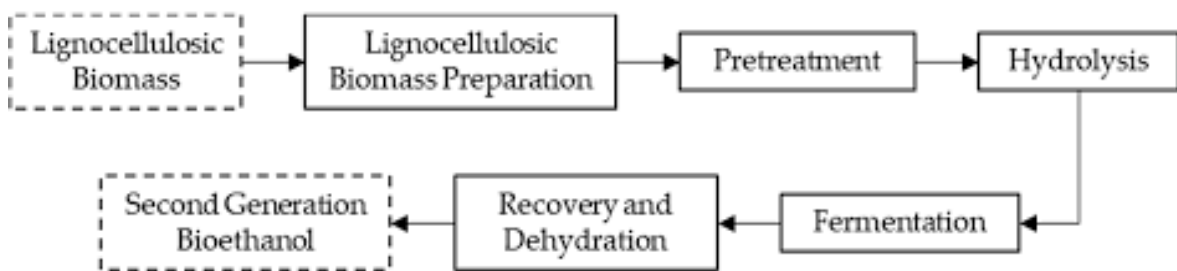


**Figure 2.6:** Steps for the conversion of lignocellulosic biomass into bioethanol (Cited from Branco et al., 2019).

Production of bioethanol is mainly influenced by the lignocellulosic contents in raw materials. According to Olsson and Hahn-Hägerdal (1996), there are some main problems encountered in the efficient conversion of the lignocellulosic hydrolysates to ethanol. Firstly,

the hydrolysate may contain other compounds after completion of the pretreatment step. For example, the hydrolysate contains not only fermentable sugars but also a broad range of compounds which has inhibitory effects on the microorganisms used for fermentation. The composition of these compounds typically depends upon the type of lignocellulosic biomass used. Apart from that, it depends also on the chemistry and nature of the pretreatment process for the fermentation. Secondly, the hemicellulose hydrolysates may contain not only hexoses but also pentoses. Hexoses are reported to be easily fermented by common yeasts such as *S. cerevisiae*. However, the pentoses are reported to be more difficult to ferment.

Based on previous research, SSF technology has been said to be more advantageous than SHF technology due to several reasons. For instance, SSF has managed to reduce the number of processes involved (Lim, 2004). Besides, SSF technology has reduced the end-product inhibition because of the rapid conversion of glucose into ethanol by yeast (Viikari et al., 2007), and reduced contamination by unwanted microorganisms due to the presence of ethanol produced in the fermentation (Elumalai & Thangavelu, 2010). According to Wright et al. (1988), he stated that although the temperature during the SSF process has to be reduced below the optimum in order for the cellulase alone to accommodate the lower temperature tolerance of fermentative organisms, the whole process of SSF actually giving the better performance especially in terms of rates, yields, and concentrations of ethanol if compared to hydrolysis alone at higher temperatures that are optimum for cellulose hydrolysis. It was reported by Ohgren et al. (2007) that ethanol yield from SSF is 13% higher than SHF process.

The idea of SSF was introduced by Gauss et al., in a patent from 1976 where it was reported by the author that the glucose yield in a traditional separate enzymatic hydrolysis was low,

which probably due to end-product inhibition of the hydrolysis by glucose and cellobiose. The author then managed to show that they obtained a higher overall ethanol yield when using the idea of SSF. According to Takagi et al. (1977), it is a process which combines enzymatic hydrolysis of cellulose or starch with the simultaneous fermentation of its derived sugars to ethanol. Since the process of enzymatic hydrolysis of cellulose or starch is combined with the fermentation, the cost of the whole process can be cut down and shorten the time. This is also supported by Sun and Cheng (2002), where he revealed the other advantages from SSF which including lesser employment of enzymes, faster production period, and less number and volume of reactors needed. Thus, lower the overall cost since both sugars releasing and sugars fermenting processes are commenced simultaneously. Figure 2.7 shows the example of SSF process where the saccharification and fermentation are conducted in the same vessel.

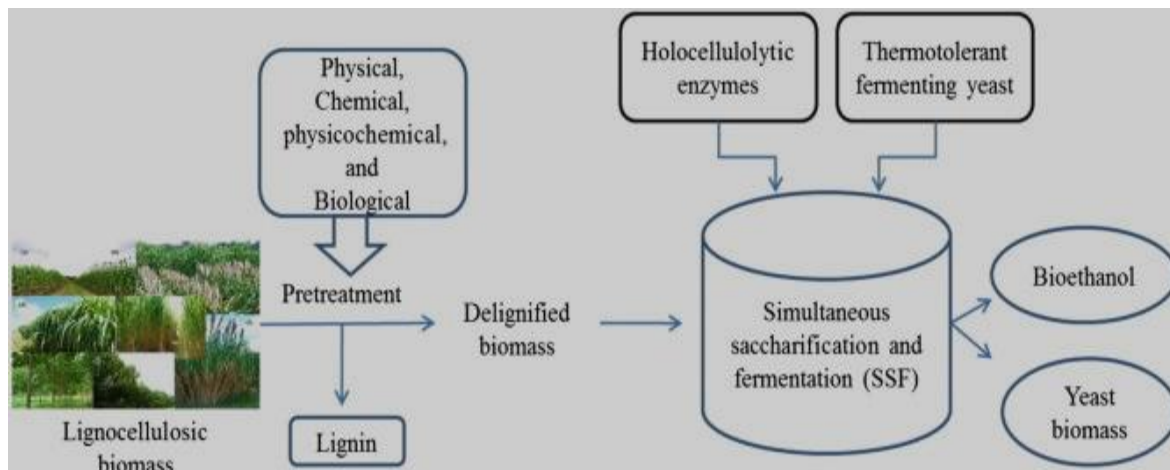


**Figure 2.7:** Bioethanol production by simultaneous saccharification and fermentation (SSF) process (Cited from Choudhary et al., 2016).

A previous study was conducted where ethanol was extracted from oil palm frond by using SSF method and it was reported that the experiment resulted in 0.32g- ethanol/g-glucose, or about 62.75% of the theoretical ethanol yield (Aditiya et al., 2016). According to Lin and

Tanaka (2006), the accumulation of ethanol in any fermentor does not inhibit cellulase as much as high concentrations of glucose, so it was mentioned that SSF is an effective strategy to increase the overall rate of cellulose to ethanol conversion. In comparison to the process where these two stages are sequential, the SSF method was previously reported that this process achieving a higher yield of ethanol which is up to 40% of yields of ethanol by removing end-product inhibition, and also by eliminating the need for separate reactors for saccharification and fermentation (Bollók et al., 2000; Stenberg et al., 2000; Hari et al., 2001).

According to Chiaramonti (2007), the hydrolysis process can be divided into two parts which are liquefaction and saccharification. In the first part, the liquefaction process utilizes  $\alpha$ -amylase enzyme, whereas the second part which is the saccharification process, utilizes glucoamylase enzyme. Meanwhile, another type of enzymatic hydrolysis is the saccharification of cellulose using cellulase. It describes a process that occurs which degrades cellulose to sugars, and it is performed by cellulolytic enzymes (produced and secreted by fungi), which allows the process to be carried out in milder conditions than acid hydrolysis. In short, enzymes hydrolysis offers the great advantage of producing higher yields of sugars with little degradation (Mussatto et al., 2010).

However, it is a big challenge to control the solution environment in SSF since both enzymes and microorganisms are employable only within the preferred temperature and pH. Huang et al. (2005) stated that most enzymes can still be comfortable with a mild acid environment (pH 5) and at the same time able to resistant to more than 313 K of temperature, although

those conditions might harm the microorganisms' sustainability.

### 2.8.1 Enzymatic hydrolysis of starchy biomass

Glucose molecular structure in starch is linked with  $\alpha$ -glucosidic bonds, while in cellulose the glucose structure is linked by  $\beta$ -glucosidic bonds. Starch is composed of a linear  $\alpha$ -1,4-linked glucans (amylose) and the same linear linkage branching with  $\alpha$ -1,6 linkages (amylopectin) (Figure 2.8) (Horváthová et al., 2000). According to Maarel et al. (2001), amylose is a linear glucose polymer with glucose unit up to 6000 units, while amylopectin is much shorter of 10–60 glucose units where the  $\alpha$ -1,6 linkage branch contains 15 - 45 glucose units. Amylose can form inclusion complexes with lipids, alcohols and iodine (Zobel, 1988). A coloured complex is formed after reaction with iodine and the colour intensity and wavelength for maximal extinction ( $\lambda_{\text{max}}$ ) depend on the concentration and average chain length of amylose (Bailey & Whelan, 1961; Banks et al., 1971).

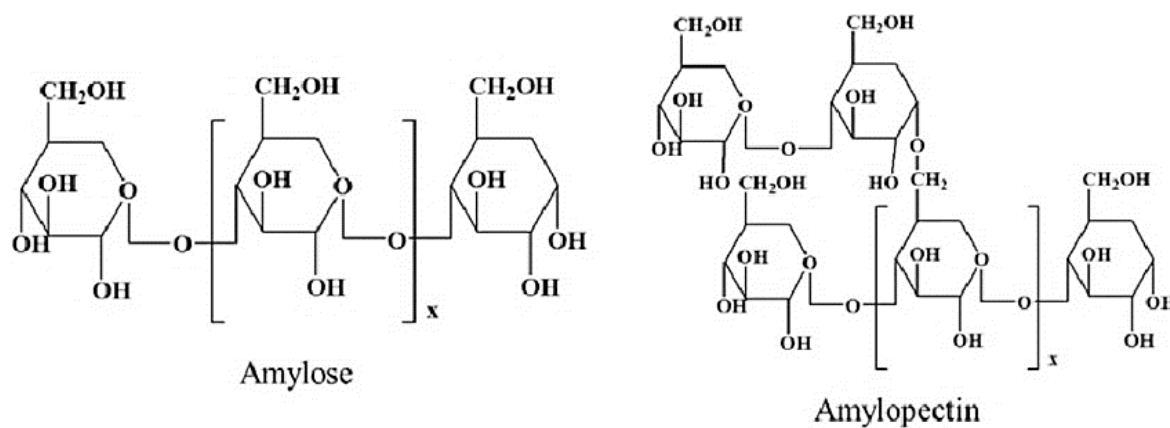


**Figure 2.8:** Structure of amylose and amylopectin (Cited from Horváthová et al., 2000).

During the saccharification step, enzymes are crucial to hydrolyze complex compounds found in lignocellulosic materials. Amylases or also called as amylolytic enzymes are the groups of enzymes that can be divided into four according to the functionality, which are endoamylases, exoamylases, debranching enzymes and transferases. The main function of these enzymes is to degrade glucose linkages in starch (Guimarães, 2012). During the breakdown of starch, these enzymes are essential. The complete breakdown of starch is crucial to the microbial industrial production of biofuels and this is currently accomplished by  $\alpha$ -amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3) during the fermentative processes (Figure 2.9) (van Zyl et al., 2012).

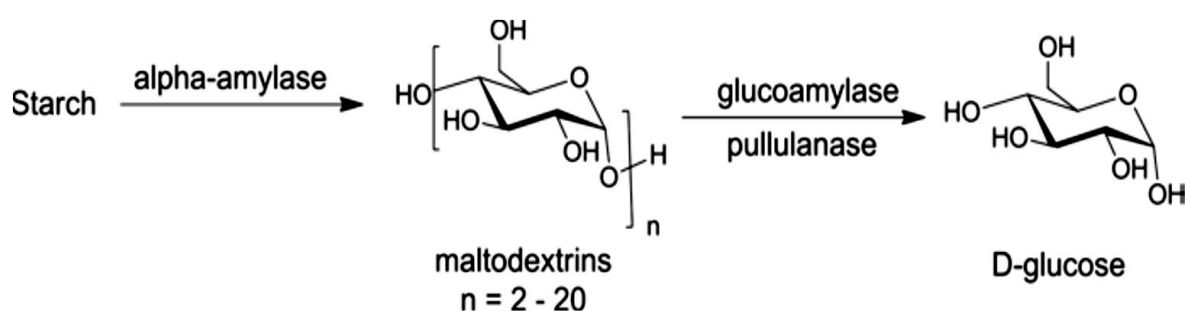


**Figure 2.9:** Enzymatic hydrolysis of starch (Cited from Sheldon, 2017).

According to Gupta et al. (2013), the amylase-catalyzed hydrolysis of starch is described as the most important industrially applied enzyme reaction. Amylases or also known as glycoside hydrolases (GHs) will act upon the bonds between the glucose units of the starch polymers. Amylases are mainly randomly hydrolyzing amylose and amylopectin (Bijttebier et al., 2008). However, the starch degrading enzymes are found in only a few of the numerous GH families, in which mainly in GH family 13, and some in GH family 14 and 15 (Henrissat, 1991; Coutinho & Henrissat, 1999). Some of the examples of enzymes which from GH

family 13 are  $\alpha$ -amylase (EC 3.2.1.1), maltogenic  $\alpha$ -amylase (EC 3.2.1.133), pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) (Coutinho & Henrissat, 1999).

Enzymes from GH family 13, or also called the  $\alpha$ -amylase family, are widely known as the enzymes that hydrolyze and/or transglycosylate  $\alpha$ -(1-4) and/or  $\alpha$ -(1-6) glucosidic linkages. According to Kuriki and Imanaka (1999), these enzymes have conserved aspartate, glutamate and aspartate residues in the catalytic site. These enzymes are also known as endo-acting amylases. During the process of starch hydrolysis, oligosaccharides will be formed with varying chain length and different  $\alpha$ -limit dextrins containing  $\alpha$ -(1-6) bonds. It stated that  $\alpha$ -amylases show also a weak transglycosylation of  $\alpha$ -(1-4) bonds (Kuriki & Imanaki, 1999).

Glucoamylases are the members of GH family 15, in which these enzymes are generally multi-fandom enzymes that consist of a catalytic domain folded as a twisted  $(\alpha/\alpha)_6$  barrel with a central funnel-shaped active site connected to a starch binding domain. These enzymes are inverting exo-amylases which releasing  $\beta$ -glucose from the non-reducing ends of starch with a single displacement mechanism. The main function of these enzymes is to hydrolyze  $\alpha$ -(1-4) bonds and  $\alpha$ -(1-6) glucosidic bonds (Pazur & Ando, 1960).

## **2.8.2 Enzymatic hydrolysis of lignocellulosic biomass**

Lignocellulosic is majorly composed of cellulose, hemicellulose and lignin, which can be degraded with enzymes to the major constituents. The acetal linkage is known as beta ( $\beta$ ),

which makes it different from starch. Another group of enzymes that crucial during enzymatic hydrolysis is cellulase. According to Henrissat et al. (1998), cellulases are described to be composed of independently folding, structurally and functionally discrete units, named as domains which producing cellulases module. A free single cellulase compound is composed of a Carbohydrate Binding Domain (CBD) at the C-terminal joined by a short poly-linker region to the catalytic domain at the N-terminal. Hydrolysis of cellulose by cellulases is controlled by two modes of action, either inversion or retention of the configuration of the anomeric carbon (Karmakar & Ray, 2011).

According to cellulases enzymatic activity, the classification is branched according to the specific task such as endoglucanase (EC3.2.1.4), exoglucanase (EC3.2.1.91) and  $\beta$ -glucosidase (EC3.2.1.21). These three major groups have specific functions in hydrolyzing cellulose. Cellulolytic enzymes act upon  $\beta$ -1, 4-glycosidic bonds of the cellulosic network by either endocleavage or exocleavage. Exoglucanases preferentially hydrolyze crystalline regions, while endoglucanases hydrolyze soluble and amorphous portions of the cellulosic network. Generally, this molecule enzyme has a similar structure, via the catalytic domain, cellulose-binding domain (CBD) and the connecting bridge (linker). As a result, cellulose can be degraded to glucose with this enzyme in synergistic action. Synergistic action of these enzymes is crucial and important for the complete hydrolysis of cellulose (Pothiraj et al., 2006).

The first enzyme, which is endoglucanase will attack low crystallinity regions in the cellulose fibres by the action known as endoaction, and thus creating free chain-ends. This

enzyme will generate oligosaccharides with different lengths and then will expose the new chain ends by cutting at the inner sites of the amorphous region of the polysaccharide chain randomly (Soni et al., 2018). The second enzyme is exoglucanases or also called as cellobiohydrolases which hydrolyze the 1, 4-glycosidyl linkages to form cellobiose. These enzymes are catalyzing nonreducing or reducing ends of the polysaccharide chain. Soni et al. (2018) in his paper stated that it is further categorized into two groups, according to the main product obtained due to its reaction. For example, if it liberates glucose, it is termed as glucanohydrolase whereas if it liberates cellobiose, it is termed as cellobiohydrolase. The products of endoglucanases and exoglucanases which are cellodextrins and cellobiose are known to be an inhibitor for the enzymes' activity. Therefore, efficient cellulose hydrolysis requires another enzyme, known as  $\beta$ -glucosidases. The presence of these enzymes will cleave the final glycosidic bonds and thus producing glucose as the end product (Karmakar & Ray, 2011).  $\beta$ -glucosidases is inactive against both amorphous and crystalline cellulose. It prefers the nonreducing ends and produces glucose by hydrolysis of cellodextrins and cellobiose (Soni et al., 2018). In detail, it subdivides  $\beta$ -1,4-endoglucanases (EG I, II, III and V),  $\beta$ -1,4-cellobiohydrolases (CBH I and II), xylanases (XYN I and II),  $\beta$ -glucosidase,  $\alpha$ -L-arabinofuranosidase, acetyl xylan esterase,  $\beta$ -mannanase and  $\alpha$ -glucuronidase (Lenting & Warmoeskerken, 2001). The whole mechanism is illustrated in the Figure 2.10.

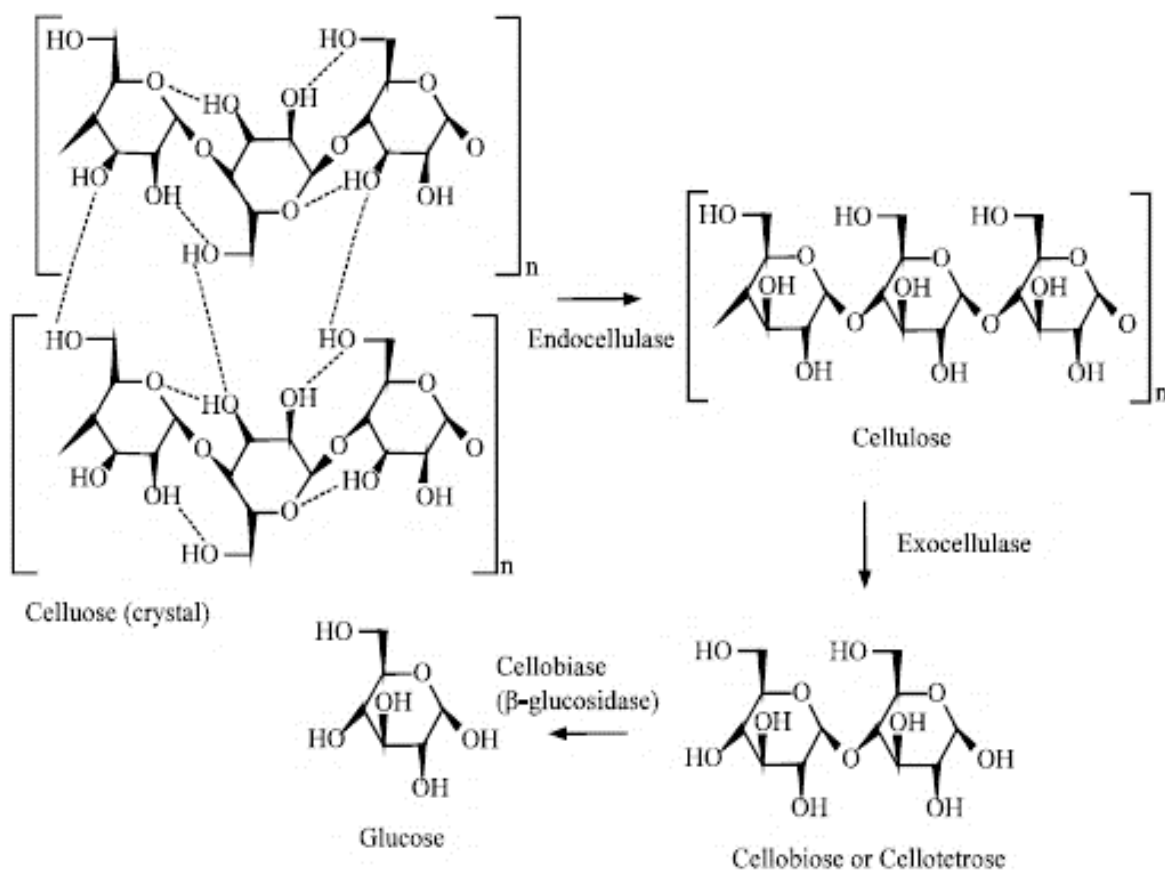


**Figure 2.10:** Mode of action of cellulases (Cited from Karmakar & Ray, 2011).

Basically, the order of the main steps involved in cellulose degradation by cellulolytic enzymes is started from binding of cellulase to the substrate via cellulose-binding domains (CBD), the followed by recognition of the susceptible bonds by cellulases for hydrolysis (exoglucanases and  $\beta$ -glucosidase recognize nonreducing ends, whereas endoglucanases recognize internal bonds of the amorphous region). The next step is the initiation of substrate hydrolysis by the formation of the enzyme-substrate complex and followed by the forward movement of the enzyme on the cellulose chain and hydrolysis of  $\beta$ -(1, 4) glycosidic bond simultaneously. Lastly, is the hydrolysis of cellobiose into glucose by  $\beta$ -glucosidase (Kumar et al., 2009; Arantes & Saddler, 2010).

Hemicellulose is said to be typically easier to hydrolyze than cellulose due to its more amorphous property (Adhitiya et al., 2016). Hemicelluloses consist mainly of other polysaccharides, such as xylans and mannans. The main feature should be made between endo- $\alpha$ ,4- $\beta$ -xylanase and xylan 1,4- $\beta$ -xylosidase (Figure 2.11). Oligosaccharides will be produced first from the cleavage of xylan and the oligosaccharides will produce xylose (Jeffries, 1994).

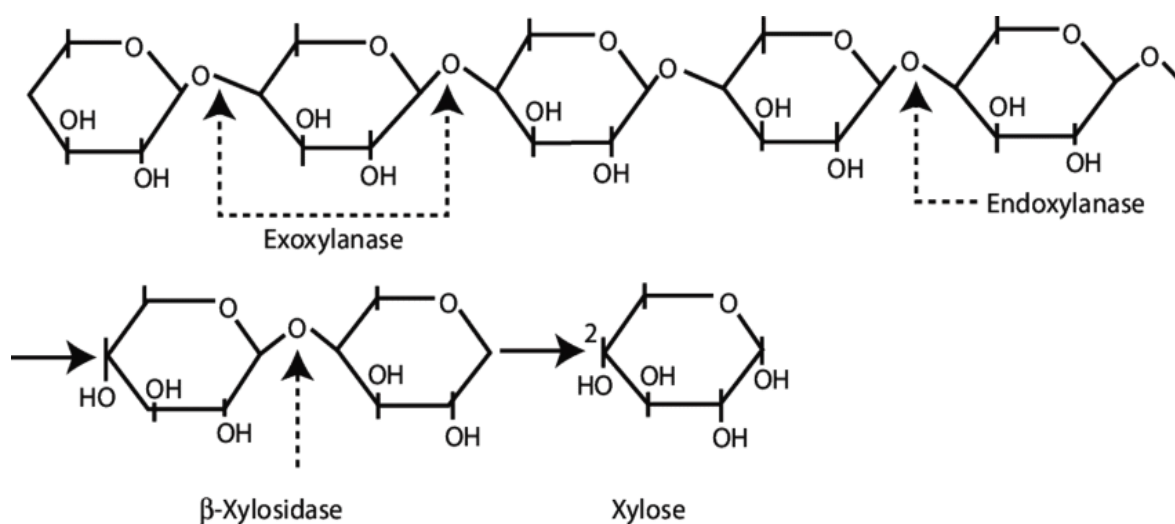


**Figure 2.11:** Enzymatic breakdown of hemicellulose (Cited from Chandra et al., 2015).

The reaction of enzymatic hydrolysis of hemicellulose requires some enzymes such as endo-1,4-xylanase,  $\beta$ -xylosidase,  $\alpha$ -glucuronidase,  $\alpha$ -arabinofuranosidase, and acetyl xylan esterase. These enzymes are responsible for the xylan degradation and saccharification (Saha, 2004; Carvalheiro et al., 2008) while the other enzymes like mannanase and mannosidase are required to cleave the glucomannan polymer backbone (Kumar et al., 2008).

## 2.9 Sago palm

Sago palm or *Metroxylon sagu* (Rottboll) (Figure 2.12), belongs to the Palmae family and is considered as an important starch producing crop distributed in the South Pacific Islands (Karim et al., 2008). Sago palm belongs to the order Arecales Nakai, family Palmae Jussieu, subfamily Calamoideae Griffith, tribe Calameae Drude, subtribe Metroxylinae Blume, and genus *Metroxylon* Rottboell (Karim et al., 2008). In general, sago palm can be found in tropical lowland forest in (Flach, 1997). Karim et al. (2008) also stated that sago palm plantations are mainly concentrated in most tropical countries. According to Yacob et al. (2018), the sago palm is said to be more beneficial crops because it is economically acceptable and relatively sustainable. Furthermore, it also promotes a socially stable agroforestry system. Malaysia ranks in the top 3 in terms of hectarage, according to the data collected which was established in 2014 (Table 2.4) (Ahmad, 2014).



**Figure 2.12:** Sago palm tree or known as *Metroxylan sagu* (CRAUN Research, 2019).

**Table 2.4:** Estimated sago hectarage by countries (Adapted from Ahmad, 2014).

| Country          | Estimated sago (ha) |
|------------------|---------------------|
| Indonesia        | 2,942,278           |
| Papua New Guinea | 1,020,000           |
| Malaysia         | 59,000              |
| Thailand         | 5,000               |
| Philippines      | 3,000               |
| Other countries  | 3,000               |
| Total            | 4,032,278           |

Sago is known for its tolerance to wet growing condition (Jong, 1995). On top of that, sago has a main advantage of the ability to thrive in harsh swampy peat environment (Ruddle, 1977). Istalaksana et al. (2005) mentioned that the palm has a lifespan of 10-20 years and flowers on average at 10-13 years. The maximum height of sago can reach up to 25 m and has a diameter of 40 cm. The stem is about 20 m long and the fruits in clusters, require 24 months to fully mature. The leaflets are linear-ensiform, can reach up to 1.5 m in length while the spadix is about 3.5-4.5 m long, with the spathes which quite spineless. Sago palm can produce both pollinated (seeded) and parthenocarpic (non-pollinated) fruits (Awg-Adeni et al., 2010). According to Oates (1999), sago palm was described as hapaxantic (only flower once in its lifetime). According to Flach and Schuiling (1989), before flowering, a sago palm trunk that averaged 1000 kg accumulated an average maximum starch content of about 150 kg. This is the average starch content acquired after analyzing several hundreds of sago palm trunks in West Malaysia.

In Malaysia, sago palm plantations are mostly found in Sarawak and it grows well in the swampy area (Yacob et al., 2018). According to Kueh (1977), sago palm can be found throughout the coastal belt of Sarawak but is concentrated in the river areas in Mukah District, Third Division. Awg-Adeni et al. (2010) stated that the world's first large-scale

commercial plantation, which about 7,700 ha near Mukah was developed by the Sarawak land development agency. According to Manan et al. (2003), 8 sago factories are operating in Sarawak, in which one of them is in Igan Sibu area, while the rest are in the Mukah and Dalat areas. In fact, sago plantation in Sarawak can produce up to 25 tons per hectare of starch in a year (Singhal et al., 2008). Based on the report from the Department of Statistics Malaysia, (2005) the export revenue of about 45,300 metric tons of food-grade sago starch earning about RM40.4 million in 2005. Approximately, by 2012, all four districts in Mukah division (Mukah, Dalat, Matu, Daro) have cultivated an estimated 11,112 ha, 28,169 ha, 4,306 ha and 3,149 ha of sago, respectively (Naim et al., 2016).

Sago has many uses either in food industries or non-food industries. For example, the sago starch can be processed to various food products like sago pearls and “tabaloi”, which is a local biscuit delicacy (Flach, 1977). Apart from that, sago flour from starch has been used for making of “keropok” (shrimp crackers) (Sidaway & Balasingam, 1971). In the biotechnology field, sago starch is used in ethanol production (Haska & Ohta, 1993; Pranamuda et al., 1995; Ratnam et al., 2003). Sago starch also can be used in lactate industry based on the report by Ishizaki (2002). Apart from that, sago starch has been previously used as biodegradable plastics (Griffin, 1977; Pranamuda et al., 1998; Odusanya et al., 2000) and the potential of using sago starch as a biomass source has been studied by Wang et al. (1996).

### **2.9.1 Sago wastes**

Sago processing industries have produced tons of wastes every year and this has become one of our main concerns. In sago processing industries, after the stage of debarking, the pith

then is mashed for the extraction of sago starch. Starch then is separated from the rest of the pith through washing in water followed by settling in tanks (Phang et al., 2000). During the processing of sago starch, there are three types of by-products were generated. These including bark of sago trunk, fibrous pith residue or known as hampas and wastewater (Awg-Adeni et al., 2010). It has been estimated that about 7 tons of sago pith waste was produced daily from a single sago starch processing mill (Bujang et al., 1966) and approximately 10 – 22 tons of wastewater are discharged into the rivers per day from factories (Phang et al., 2000). These wastewater effluents are not suitable to be reused and thus the wastes are discarded into a nearby river (Bujang et al., 1996; Awg-Adeni et al., 2012). DOA (2011) stated that it is estimated more than 68 thousand tonnes of fibres, 83 thousand tonnes of barks, and 960 thousand tonnes of wastewater are generated annually.

### **2.9.2 Total sago effluent**

Total sago effluent is a mixture of sago hampas residues and sago effluent hydrolysate. It is considered as biomass as it contains lignocellulosic materials like cellulose, hemicellulose and lignin. These lignocellulosic components are suitable for combustion for renewable energy production and bioethanol production as it can be converted into fermentable sugars such as glucose (Singhal et al., 2008). Sago hampas is a starchy lignocellulosic. It was reported that the cellulose fraction mostly consisted of glucose which about 89% and the remaining are small amounts of other sugars like xylose, rhamnose, arabinose, mannose, fucose and galactose. Meanwhile, the major components found in isolated hemicelluloses are xylose and glucose, along with noticeable amounts of arabinose and galactose and also small amounts of rhamnose, mannose, fucose and uronic acids (Awg-Adeni et al., 2010). In

another study by Phang et al. (2000), it was reported that the sago hampas contains up to 66% fermentable carbohydrate, which beneficial and may be used as animal feed, compost for mushroom culture, hydrolysis to confectioners' syrup and particleboard manufacture. On the other hand, sago effluent was previously reported to contain high carbon to nitrogen ratio (105:0.12), which makes it a suitable substrate for fermentation and the production of high protein fungal biomass (Singhal et al., 2008).

Nowadays, many studies have been conducted to utilize sago wastes for other uses. The utilization of sago residue into other beneficial products is expected to reduce the amount of accumulation of sago wastes and help to minimize the rate of pollution. For instance, these sago wastes can be exploited commercially to produce value-added end-products. For example, fermentable sugar, enzymes, adsorbents and bioethanol (Singhal et al., 2008; Linggang et al., 2012; Awg-Adeni et al., 2013). Several studies on the compositional analysis of sago hampas have been performed since sago waste is used to produce some value-added end products. It was reported that 26% of sago bark and 14% of sago hampas counted of the total weight of sago block (Singal et al., 2008). Sago waste contains about 65.7% of dissolved starch while water waste contains organic materials like starch and cellulose (Phang et al., 2000). Based on previous studies, the contents of each component are shown in the Table 2.5.

**Table 2.5:** Compositional analysis of sago hampas.

| <b>Sago hampas component</b> | <b>Linggang et al., (2012)</b> | <b>Vincent et al., (2015)</b> |
|------------------------------|--------------------------------|-------------------------------|
| <b>Starch</b>                | 58.0                           | 58.0 ± 1.2                    |
| <b>Cellulose</b>             | 23.0                           | 23.5 ± 1.0                    |
| <b>Hemicellulose</b>         | 9.2                            | 8.19 ± 0.2                    |
| <b>lignin</b>                | 4.0                            | 6.3 ± 1.0                     |

## 2.10 Microorganisms involved in fermentation

In any fermentation process, the selection of the appropriate microorganism plays a crucial role to achieve high efficiency of ethanol production. Production of ethanol can be derived from the process of fermentation which commonly uses the action of yeast (Demirbas, 2007). The desired microorganism should be capable of converting sugars in hydrolysates into ethanol with high efficiency. The process of fermentation requires the supporting conditions for the microorganism to grow and sustain such as temperature and pH (Demirbas, 2004). As for mesophilic organisms, the suitable temperature for most of the fermentation agents is within 29.85 °C – 37.85 °C (Hettenhaus, 1998). According to Aminifarshidmehr (1996), the best pH range for bacteria type of fermentation microbes is between 6.5-7.5. Meanwhile, Balat et al. (2008) reported that fungi can grow in more acidic environment of pH 3.5-5.0.

Diverse groups of microorganisms can produce ethanol as stated in previous studies (Sims et al., 2008; Soccol et al., 2010; Dias et al., 2011). According to van Dijken et al. (1986), probably most species of yeasts able to ferment sugar to ethanol. Barnet et al. (2000) stated that out of 678 recognized species, about 60% are deemed to be fermentative based on taxonomic tests which including gas production in Durham tubes. However, this range is even higher since, under certain conditions, some of those species considered as non-fermentative are also capable of fermenting glucose (van Dijken et al., 1986; Visser et al., 1990). Some of the examples are yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, bacteria like *Zymomonas mobilis*, fungus *Fusariumoxys porum*, yeast-like fungus *Pachysolen tannophylus*, and thermophilic bacteria (Chaudhary & Chincholkar, 1986). For example, *Zymomonas mobilis* ferments glucose, sucrose and fructose into ethanol. According to Mohagheghi et al. (2002), bioethanol production using

*Zymomonas mobilis* as the fermentation agent, has produced 12% (w/v) of ethanol with a theoretical yield of 97%.

### **2.10.1 Fermentative yeasts**

One of the most promising microorganisms involved in fermentation is *Saccharomyces cerevisiae* or commonly known as baker yeast. *S. cerevisiae* has been used since a long time ago in alcoholic fermentation. Kosaric and Velikonja (1995) stated that fermentation of sugar using single microorganisms such as yeast is one of the oldest practices in biotechnology, which mostly used to produce drinking alcohol, such as wine and beer. At present, this application is used to yield bioethanol from renewable energy sources. Microorganisms that considered as ethanologenic microorganisms must possess some characteristics such as, able to produce higher ethanol yield (>90.0% theoretical yield), tolerance to ethanol (>40.0 g/l) and good ethanol productivity (>1.0 g/l/h). Aside from that, those microorganisms must possess a good growth in simple and inexpensive media and at the same time have the capability of growth in undiluted fermentation broth with resistance to inhibitors (Dien et al., 2003).

According to Lin and Tanaka (2006), yeast is the most commonly used microbe and among yeast species, *S. cerevisiae* is previously reported to yield high ethanol concentration which as high as 18% of the fermentation broth. Therefore, *S. cerevisiae* is preferred for industrial ethanol production. This is mainly due to their good fermentative capacity and high tolerance to ethanol and other inhibitors. *S. cerevisiae* also able to grow rapidly under the anaerobic conditions, especially in large-scale fermentation reactors. However, Musatto et al. (2010)

stated that *S. cerevisiae* can only ferment hexose sugars such as glucose and mannose, but not pentoses such as xylose and arabinose. Microorganisms which shown to be an efficient ethanol producer from xylose in laboratory media will not necessarily able to ferment the lignocellulosic hydrolysates efficiently (Olsson & Hahn-Hägerdal, 1996). *Z. mobilis* and *S. cerevisiae* are known as the most commonly employed microorganisms in fermentation, but both were previously reported for being incapable of fermenting pentose sugars. On the other hand, other microorganisms have the capability of fermenting pentose sugars (xylose), such as *Candida shehatae*, *Pichia stipitis*, and *Pachysolen tannophilus*. Therefore, the study of the fermentative performance of microorganisms in lignocellulose is a useful way of finding suitable strains.

### **2.10.2 *Candida glabrata* as fermentative yeast**

In this study, the species of yeast used is *Candida glabrata*. *C. glabrata* can be found in ragi, which is the traditional fermented food in Sarawak (Kofli & Davaon, 2010). It was previously proposed that *C. glabrata* is suitable for bioethanol fermentation as it has higher stress tolerance to both acid and high temperatures in addition to possessing effective ethanol conversion capabilities (Watanabe et al., 2008; Watanabe et al., 2009; Watanabe et al., 2010). Thermotolerant yeast is suitable for SSF since the optimum temperature for cellulases activity is between 40 to 50 °C. This could enhance saccharification process and thus increase the ethanol yield. According to Visser et al. (1990), some of the strains of *Candida* are unable to grow and produce ethanol under anaerobic conditions. However, Merico et al. (2007) stated that *C. glabrata* had shown higher abilities of ethanol production and growth

under anaerobic conditions. Furthermore, the characteristics of *C. glabrata* were reported to be similar with *S. cerevisiae* in ethanol fermentation.

### **2.10.3 Characteristics of *C. glabrata***

In 1917, *C. glabrata* was named as *Cryptococcus glabratus* and known as a component of the human gut microbiota (Anderson, 1917). Then, it was named as *Torulopsis glabrata* (Grimley et al., 1965) which is a common non-hyphae forming yeast isolate in the clinical laboratory. *C. glabrata* was firstly classified under genus *Torulopsis* due to its lack of pseudohypha production but in 1978, it was confirmed that the ability to produce pseudohyphae was not a reliable distinguishing factor for members of the genus *Candida* and thus it was classified as a member of *Candida* genus, along with over 200 species (Kwon-Chung & Bennett, 1992). According to previous studies, *C. glabrata* has been considered a relatively nonpathogenic saprophyte of the normal flora of healthy individuals, in which it was rarely causing severe infection in humans (Haley, 1961; Stenderup & Pederson, 1962).

Kurtzman and Robnett (1998), reported that *C. glabrata* is similar to *S. cerevisiae* and stated as a close relative to *S. cerevisiae*. *C. glabrata* has been added together with other *Saccharomyces* species to the joint *Saccharomyces* clade (Kurtzman & Robnett, 1998). This also supported by other papers in which they stated that *C. glabrata* is considered a close relative of *S. cerevisiae* based on the genome evolution (Dujon et al., 2004; Pikur & Langkir, 2004; Herrero, 2005; Nakagawa & Mizuguchi, 2005). However, it was reported that the mechanisms of stress response of *C. glabrata* may be different from *S. cerevisiae* and based

on the study by Gregori et al. (2007), he pointed out the differences high osmolarity, between *C. glabrata* and *S. cerevisiae*.

The size of the cell was reportedly to be small compared to other *Candida* species and is in the range of 1 to 4  $\mu\text{m}$  (Fidel et al., 1999). The clear image of *C. glabrata* cells under the microscope is shown in the Figure 2.13. According to Whelan et al. (1984), an important distinguishing characteristic of *C. glabrata* is the haploid genome, which diverse from *Candida. albicans* and several other non-albicans that have a diploid genome. *C. glabrata* was stated as the second most commonly isolated yeast species in clinical laboratories (Fenn et al., 1999; Peltroche-Llacsahuanga et al., 1999).

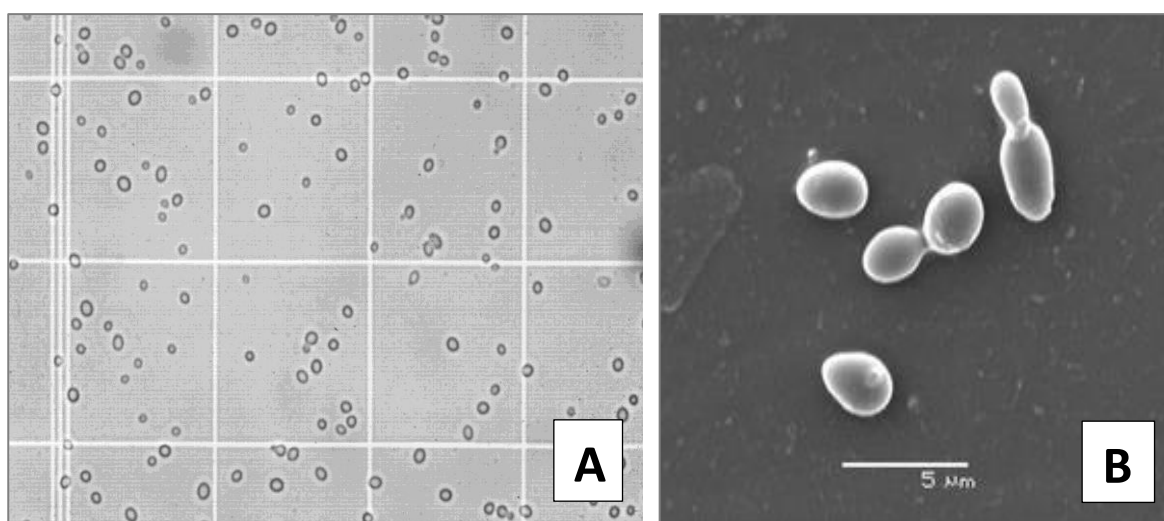


**Figure 2.13:** *C. glabrata* yeast cells (A) on a haemocytometer, magnification 400x (A) (Cited from Fidel et al., 1999); (B) Electron microscope (Cited from Ahmad et al., 2014).

The biochemical reactions of *C. glabrata* are completely distinct if compared to other *Candida* species. For instance, *C. glabrata* only capable of fermenting glucose and trehalose unlike *C. albicans* that capable of fermenting several sugars (Bennet & Kwon-Chung, 1992).

This also supported by Barnett et al. (2000), where he also reported that *C. glabrata* can only ferment two type of sugars. This became the critical factor that distinguishes this yeast from most of the other yeasts that in general ferment various type of sugars. These sugars are glucose and trehalose. Trehalose is disaccharide sugar which important for several microorganisms (including fungi), plants and insects (Elbein et al., 2003).

## **2.11 Carbohydrate and ethanol analysis**

### **2.11.1 HPLC analysis**

The analysis of carbohydrates and their conjugates is one of the most requested analyses at both biochemical and nutritional analytical laboratories as quoted by Yamada and Kakehi, (2011). The product of fermentation which containing carbohydrates can be analyzed further by using other analysis. For example, the concentration of specific sugar can be quantified by using High Performance Liquid Chromatography (HPLC) analysis (Shaw, 1988; Kelebek et al., 2009; Nefolar et al., 2010).

HPLC is an instrument widely used in the determination of specific carbohydrates contents. According to Synder et al. (2010), HPLC is used to separate compounds in a mixture by using the compounds different partition properties between a stationary phase and a mobile phase. The main components include a pump, injector, column, detector and integrator and display system, in which the most vital component is the column where separation occurs (Kupiec, 2004) (Figure 2.14). Separation of components occurs when analytes and mobile phase are pumped through the column. Kupiec (2004) also mentioned that apart from separation, detection of the eluting components also important. On top of that, the chosen

method for detection is dependent upon the detector used. The suitable type of detection that used for carbohydrates analysis is refractive index detection or often called as RID (Bruno & Krattiger, 1995). RI detector is often used due to the simplicity of analysis and the costs for equipment and reagents are considerably lower than other techniques (Ellefson, 2003). According to Yan (2016), the greater the refractive index differences between the effluent and the mobile phase, the greater the whole signal. RID was also reported to have a high detection limit and poor selectivity. The main principle of RID is to measure the change in the refractive index of effluent passing through the flow-cell relative to the mobile phase.

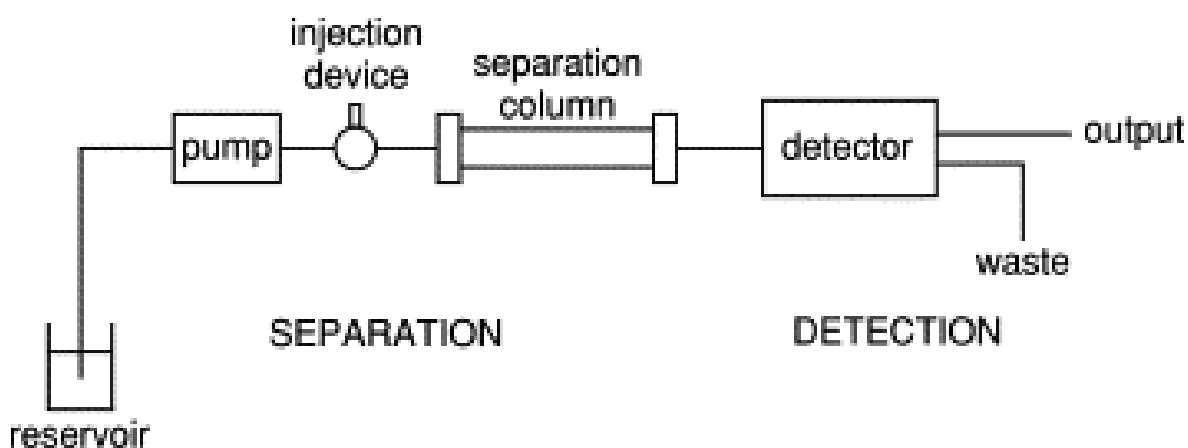


**Figure 2.14:** Schematic diagram of a typical HPLC system (Cited from Forga'cs & Cserha'ti, 2003).

Yan (2016) also stated that the separation can be controlled. This can be done either by adjusting the concentration of the mobile phase or adsorption properties of the stationary phase. Generally, types of separation are categorized according to the type of stationary and mobile phases used. For example, in Normal Phase HPLC, the separation is based on polarity, in which the stationary phase is polar like silica while the mobile phase is non-polar solvent. Meanwhile, in Reverse Phase HPLC, the principle is opposite with Normal Phase

where the mobile phase is polar and the stationary phase is nonpolar or hydrophobic (Thammana, 2016).

### **2.11.2 Phenol sulfuric acid (PSA analysis)**

Other than HPLC, another method for the detection of carbohydrates contents is using phenol-sulfuric acid assay or PSA (Dubois et al., 1956). This method does not require hydrolysis of the carbohydrates and can detect mono-, oligo-, and polysaccharides (Nielsen, 2010). PSA method can be described as a relatively simple and low-cost analysis method for sugar concentration estimation. Basically, the main principle of phenol-sulfuric acid assay is that when the carbohydrates dehydrated by a specific reaction together with the concentrated sulfuric acid, it will then produce furfural derivatives at the end of the reaction (Dubois et al., 1956; Chow & Landhäusser, 2003; Asghari & Yoshida, 2006; Albalasmeh et al, 2013). Nielsen (2010) explained that concentrated sulfuric acid will break down the large molecule of any polysaccharides, oligosaccharides and disaccharides to yield monosaccharides as end-product. For example, pentoses (5-carbon compounds) will be dehydrated to furfural, while hexoses (6-carbon compounds) will be dehydrated to hydroxymethyl furfural. Later, these compounds will react with phenol to produce a yellow-gold colour.

For the preparation of the standard curve, when the products or samples are high in hexose sugars (6-carbon compounds), glucose is used for the standard curve, and the absorbance is recorded at 490 nm. Meanwhile, for products or samples that are very high in xylose which is a pentose sugar (5-carbon compounds), it is recommended to use xylose to construct the

standard curve for the assay and the absorbance is measured at 480 nm. The colour of this reaction is stable for a few hours, and the accuracy of this assay is reported to be within the range of  $\pm 2\%$  under proper conditions (Nielsen, 2010).

## CHAPTER 3

### METHODOLOGY

#### 3.1 Experimental setup

Figure 3.1 shows an overview of the experimental procedures in this study. The first step was preparation of glycerol stock of *Candida glabrata*. From the glycerol stock, yeast inoculum was prepared for the fermentation purpose. Next, biomass (total sago effluent, TSE) was collected, and compositional analysis of biomass was conducted. Then, fermentation was carried out by batch, based on type of substrates, as shown in Figure 3.1. Lastly, sample from fermentation process and SSF was collected and was subjected to different analysis such as cell growth, HPLC profiling and phenol sulfuric acid (PSA).

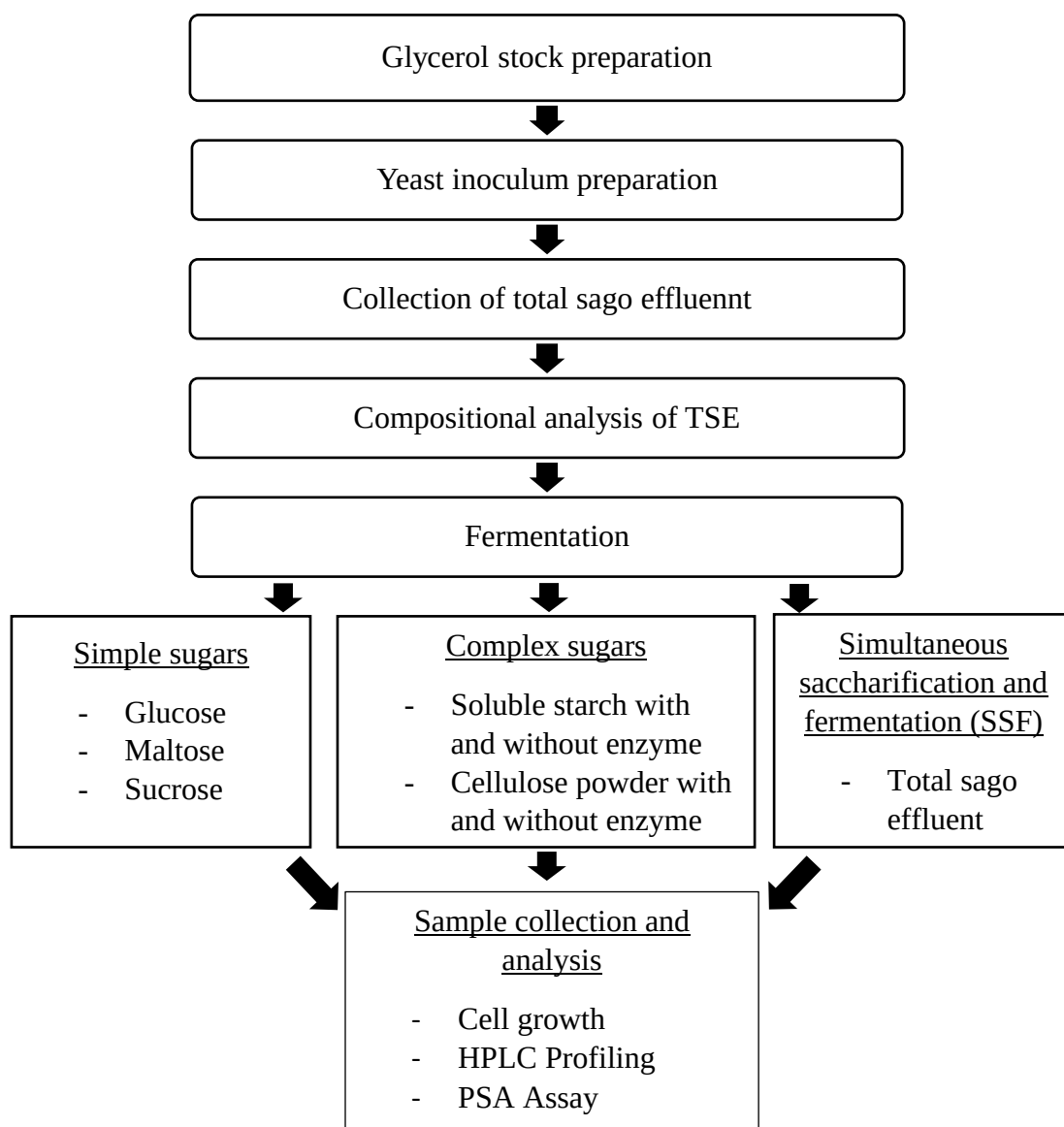


**Figure 3.1:** Flowchart of experimental design.

## 3.2 Materials and methods

### 3.2.1 Glycerol stock preparation

The *C. glabrata* strain used in this study was obtained from the stock cultures in the Microbiology lab 2 (FRST, Unimas). This strain was originally isolated from “ragi”, one of the fermentation starters in food fermentation. The stock culture of *C. glabrata* was revived

in Yeast Malt (YM) broth (Sigma, USA) and was grown overnight by shaking (NB-101MT Multi Shaker, N-Biotel, Korea) at ambient temperature with agitation speed of 150 rpm. Subcultures of selected colonies were done on the Rose Bengal Chloramphenicol Agar, RBDC (Hi Media, India) (Figure 3.2) for isolation purposes and was incubated for 48 hours. Single colonies were selected and inoculated onto YM broth and grown again for overnight. Simple staining was carried out by using crystal violet and was observed under light microscope (Omax, Korea) (Figure 3.2). For long term storage, the stock culture was prepared by mixing culture medium with addition of 20% (v/v) glycerol (R&M Marketing, UK) and the final volume of 2.0 ml were stored at -20 °C in freezer (SJC203 Refrigerator, Sharp, Malaysia) for preservation.

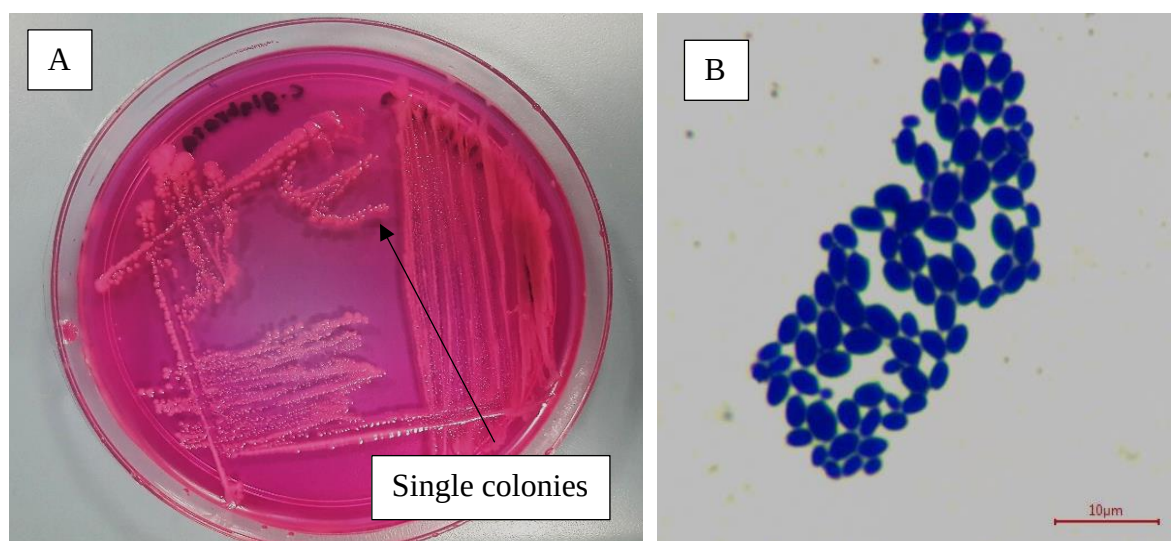


**Figure 3.2:** (A) Colonies of *C. glabrata* formed on RBDC agar; (B) Morphology of *C. glabrata* under light microscope.

### 3.2.2 Yeast inoculum preparation

For yeast inoculum preparation, the culture was taken from the glycerol stocks. For preparation of YM agar, about 2.15 g YM broth was dissolved in 100 ml distilled water. The broth prepared was sterilized by using autoclave for 15 min at 121 °C. The cultures were

grown overnight in a broth containing YM broth at ambient temperature and constant agitation of 150 rpm. The cells were harvested via centrifugation (BK-1032J Low Speed Centrifuge, Biobase, China) in two 50 ml conical centrifuge tubes for 6 min at 4500 rpm (Vincent et al., 2011). The supernatants (Figure 3.3) were discarded, and the *C. glabrata* cells pellet were transferred into fermentation broth.

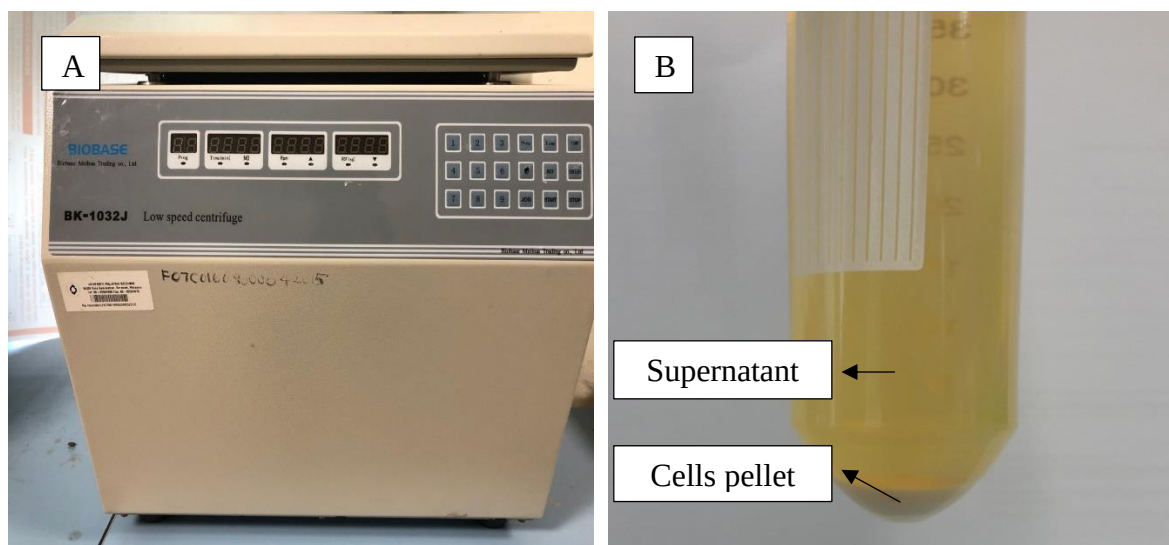


**Figure 3.3:** (A) Low speed centrifuge; (B) Harvested cells after centrifugation.

### 3.2.3 Collection of total sago effluent

Total sago effluent was collected from Herdsen Sago Mill Sdn. Bhd in Pusa, Sarawak (Figure 3.4). Then, it was stored in a PVC tank and keep in a -20 °C Prior to its usage, the tank was thawed and shaken to ensure all the effluent and sago hampas were mixed well.



**Figure 3.4:** Total sago effluent from Sago Mill, Pusa (A) Front view; (B) Side view.

### **3.2.4 Characterization of Sago hampas**

#### **3.2.4.1 Determination of moisture content**

The sample moisture content was determined by weight loss from initial weight after oven-drying (Shel Lab, USA) at 105 °C for 3 days. Prior to characterization process, the total sago effluent was destarched and oven-dried before it was ground to fine powder. The powdered form of sample was subjected to starch and fibre analysis by using the method Phenol-Sulphuric Acid assay (PSA), Acid detergent Fibre (ADF), Neutral Detergent Fibre (NDF) and Klason Lignin Determination. Determination of ash content then was determined using furnace incineration, where the sample was subjected to dry ashing at 550 °C for 6 hours in an ashing muffle furnace (KC, 40/13. ThermConcept, Germany) until ash was obtained.

### **3.2.5 Fermentation broth preparation**

#### **3.2.5.1 Simple sugars (monosaccharide and disaccharide)**

Fermentation broth was prepared in 500 ml schott bottle containing 150 ml of mixed solution of yeast extract (Bacto, USA), bacteriological peptone (Bendosen Laboratory Chemicals, Malaysia) and 5% of sugar as substrate in 0.05 M citrate buffer. The selected substrates were glucose (R&M Chemical, United Kingdom), maltose (R&M Chemical, United Kingdom) and sucrose (Bendosen Laboratory Chemicals, Malaysia). After adjusting the pH of the broth to 4.8, the media were autoclaved (Model No. 25X Electric Pressure Steam Sterilizer, All American, United State) for 15 min at 121 °C for sterilization purpose.

#### **3.2.5.2 Soluble starch and cellulose powder**

Another batch of fermentation broth was prepared in 500 ml schott bottle containing 5% of soluble starch (Bendosen Laboratory Chemicals, Malaysia) and 5% of cellulose powder (Nacalai Tesque, Japan). Yeast extract and bacteriological peptone were added into the broth and the broth was topped up to 150 ml with 0.05 M citrate buffer. Then, the pH of the broth was adjusted to 4.8 and the prepared media were autoclaved to provide sterility and to gelatinize the starch.

#### **3.2.5.3 Total Sago Effluent**

For the last batch, fermentation broth was prepared in 500 ml schott bottle containing a mixed solution of total sago effluent as main substrate and 0.05 M citrate buffer, with final volume of 150 ml. Total sago effluent was supplemented with yeast extract and

bacteriological peptone. After adjusting the pH to 4.8, the media were sterilized in an autoclave for 15 min.

### 3.2.6 Fermentation of simple sugar (glucose, maltose and sucrose)

Prior to the fermentation, the harvested cells were added aseptically into the sterilized fermentation media containing glucose, maltose and sucrose. Fermentation process was carried out for 5 days, at ambient temperature with constant agitation speed of 150 rpm. For each substrate, the fermentations were carried out in triplicates (n=3). Figure 3.5, Figure 3.6 and Figure 3.7 show the condition of fermentation broth after 5 days of fermentation.

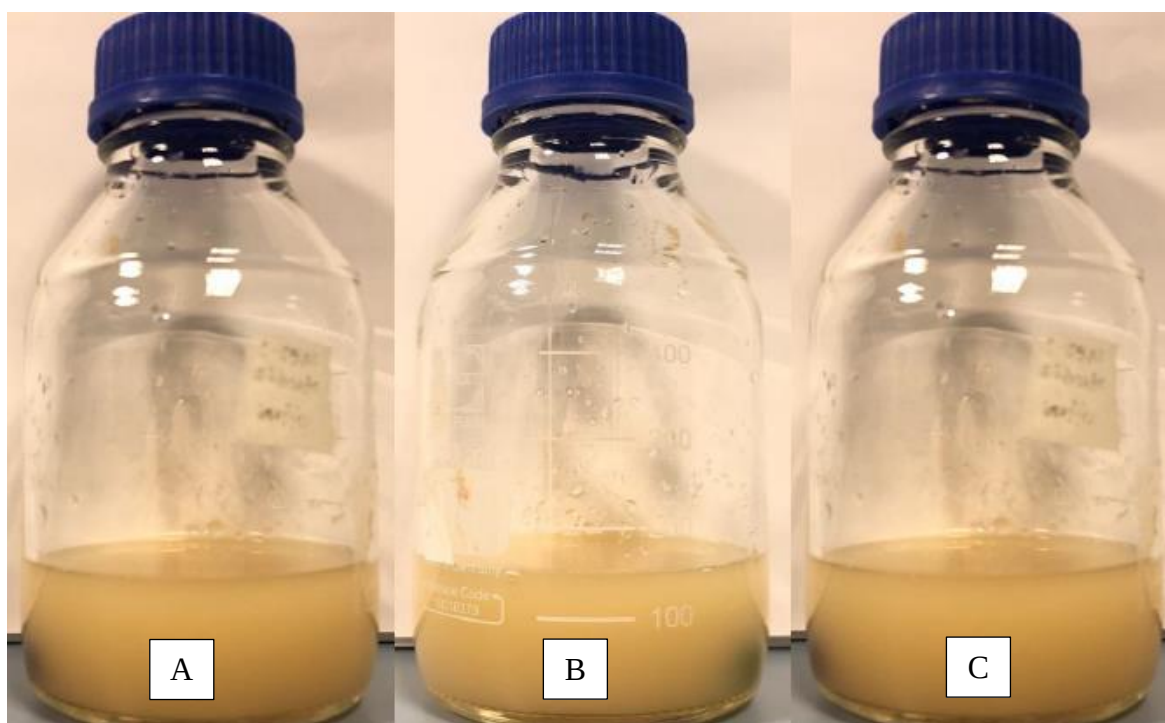


**Figure 3.5:** Fermentation broth containing 5% glucose in 150 ml total volume. All broths turned cloudy after 120 h (A) Replicate 1; (B) Replicate 2; (C) Replicate 3.

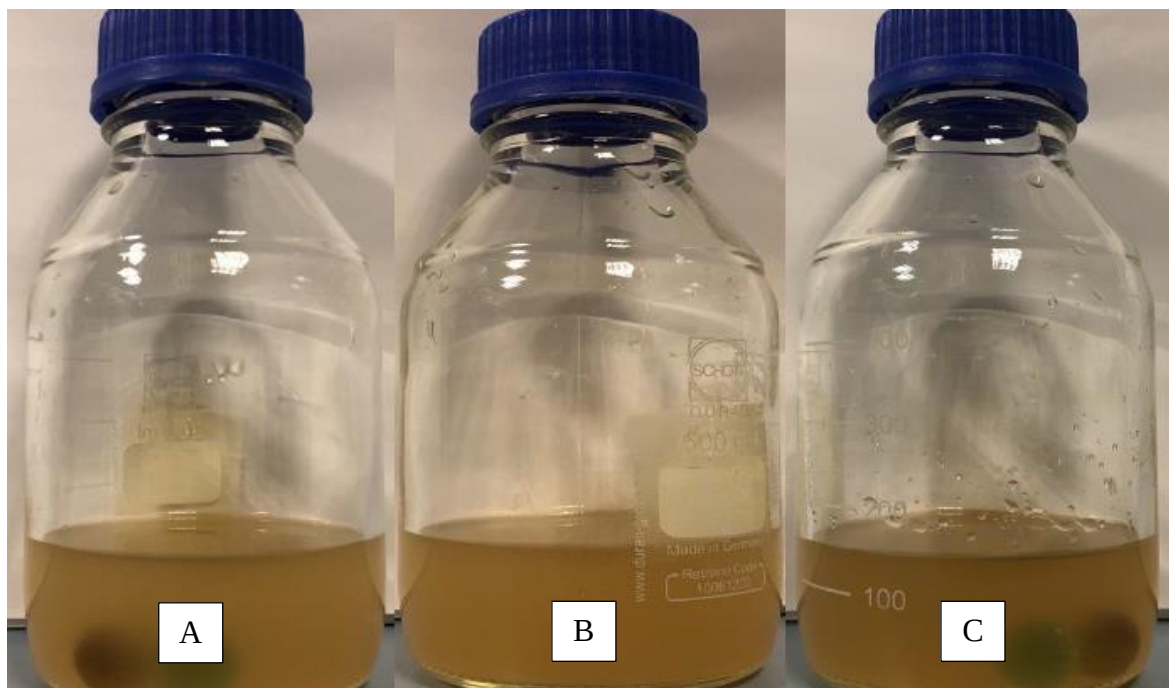


**Figure 3.6:** Fermentation broth containing 5% maltose in 150 ml total volume. There was no colour changes observed in all broths after 120 h (A) Replicate 1; (B) Replicate 2; (C) Replicate 3.

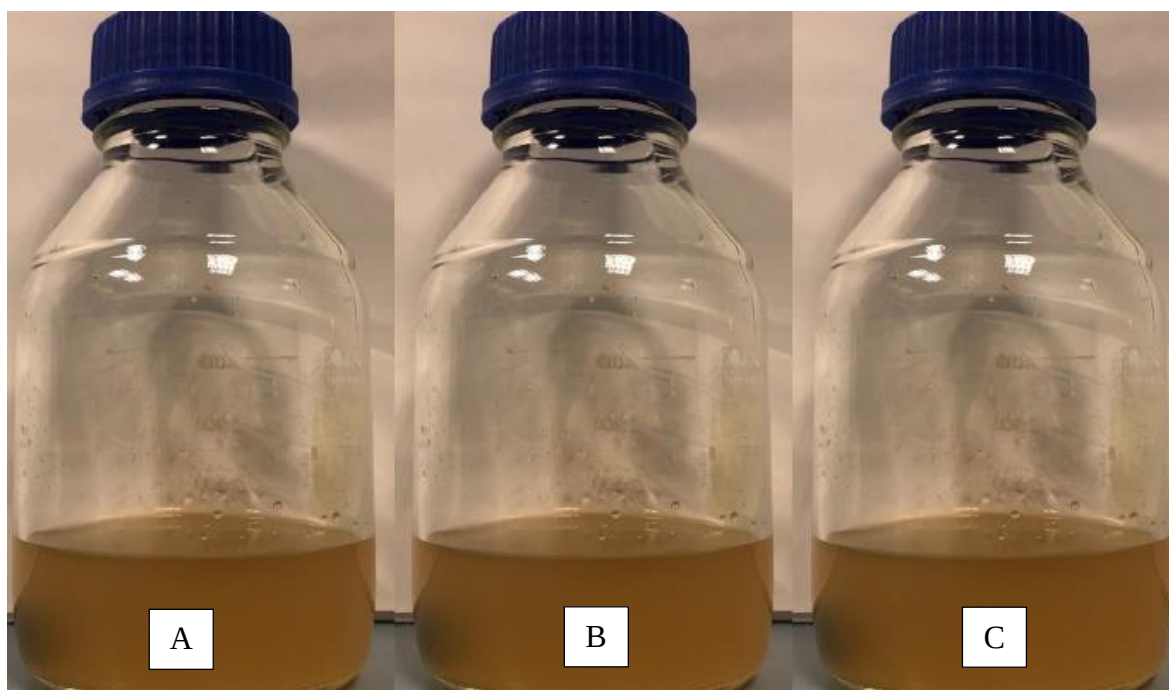


**Figure 3.7:** Fermentation broth containing 5% sucrose in 150 ml total volume. There was no colour changes observed in all broths after 120 h (A) Replicate 1; (B) Replicate 2; (C) Replicate 3.

### 3.2.7 Fermentation of soluble starch and cellulose powder (with and without enzymes)

The sterilized fermentation media was added with harvested cells before the fermentation process. Then, amylase enzymes (Sunson, China) were added into soluble starch broth and cellulase enzyme (Sunson, China) were added into cellulose powder broth. Fermentation process was conducted for 5 days, with constant agitation speed of 150 rpm at ambient temperature. Fermentation was carried out once again, containing the same substrates but without addition of enzymes. Fermentation process was conducted with the same conditions. For each batch, the fermentations were performed in triplicates ( $n=3$ ) as shown in Figure 3.8 and Figure 3.9.

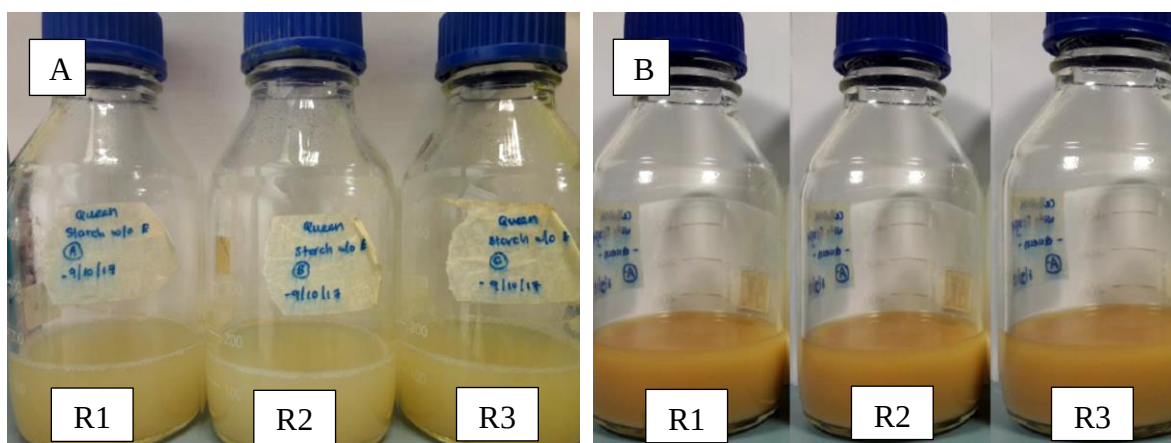


**Figure 3.8:** Fermentation broth containing 5% soluble starch, in triplicates (R1, R2, R3) (A) Soluble starch without enzyme, there was no significant changes observed in all broths after 120 h (B) Soluble starch with enzyme, the colour of the broths changed from cloudy to yellowish after 120 h.

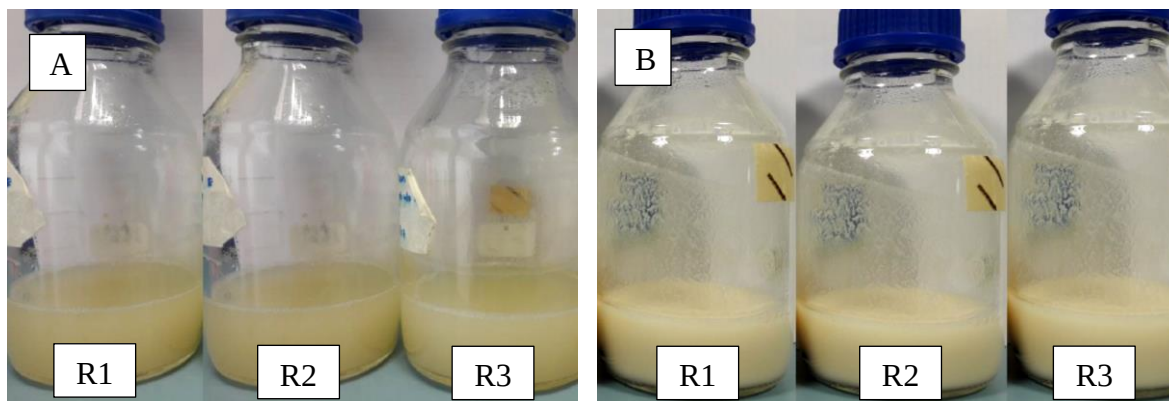


**Figure 3.9:** Fermentation broth containing 5% cellulose powder, in triplicates (R1, R2, R3) (A) Cellulose powder without enzyme, there was no significant changes observed in all broths after 120 h; (B) Cellulose powder with enzyme, the broths turned cloudy after 120 h.

### 3.2.8 Simultaneous saccharification and fermentation (SSF)

Prior to the SSF, the enzymes of amylase and cellulase were added into the sterilized fermentation media containing total sago effluent. About 0.75  $\mu\text{l}$  of each  $\alpha$ -amylase and gluco-amylase were added respectively into the fermentation broth. Then, cellulase enzyme with enzyme activity of 50 was added. After addition of enzymes, harvested cells were added into the media to start the SSF process. SSF process was conducted for 5 days, with constant agitation speed of 150 rpm and at ambient temperature. SSF were performed in triplicates ( $n=3$ ) as shown in Figure 3.10.

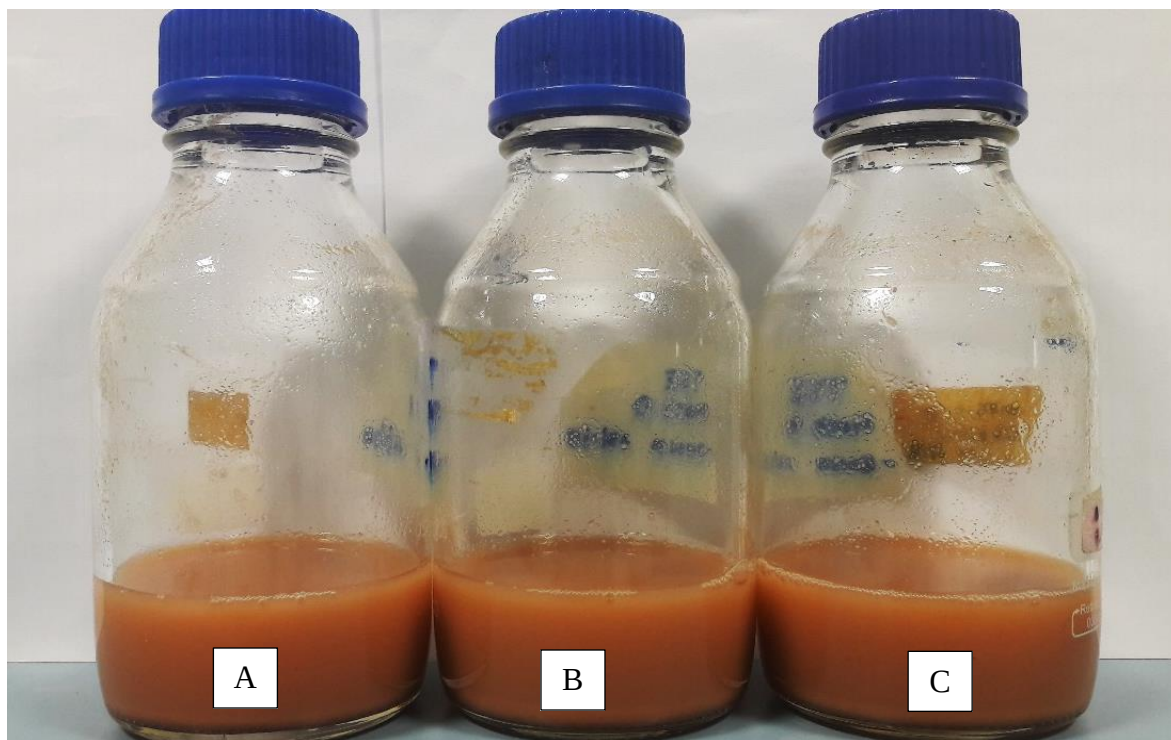


**Figure 3.10:** Fermentation broth containing 150 ml of TSE, after 120 h of SSF (A) Replicate 1; (B) Replicate 2; (C) Replicate 3.

### 3.2.9 Sample collection

For samples collection, 1.5 ml of samples were pipetted out at 0, 6, 12, 24, 36, 48, 72, 96 and 120 h. Evaluation of the cell growth was conducted for every sampling hour using the cell counting method. Meanwhile, for the HPLC analysis, the samples taken were centrifuged by using microcentrifuge (Figure 3.11.A) (*WiseSpin* CF-10 High-Performance Microcentrifuge Set, Daihan Scientific, Korea) at 13500 rpm for 3 min. Microcentrifuge helps to separate mixture in a liquid sample by spinning at high speed and thus forming pellet and supernatant. Pellet is the compressed mass of a substance, which commonly formed at the bottom of the tube meanwhile supernatant is a liquid above a solid residue after centrifugation, as shown in Figure 3.11.B. Prior to the HPLC analysis, the supernatant was

filtered through 0.45 µm nylon membrane filter (Whatman, NJ, USA) to eliminate any solid residues.

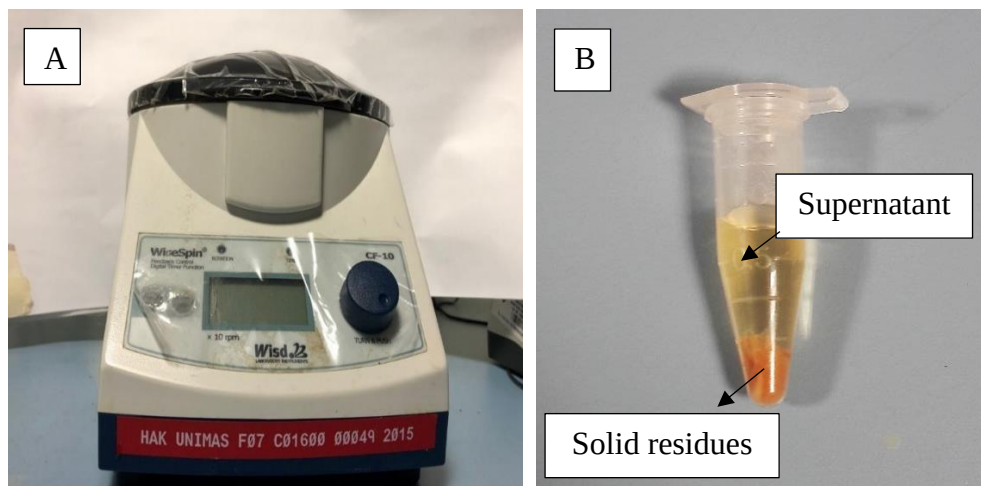


**Figure 3.11:** (A) Microcentrifuge; (B) Centrifuged sample.

### 3.2.10 Sample analysis

#### 3.2.10.1 Cell count method

Cell counting was performed by using haemocytometer (Optik Labor, United Kingdom) (Figure 3.12.A). Haemocytometer is a slide or counting-chamber device used for counting the number of viable cells in a sample. The viable cells were observed under microscope with the help of trypan blue for staining purpose (Zoecklien et al., 1995). Viable cells refer to the unstained cells, or white in color, as shown in Figure 3.12.B, with the assumption that dead cells take up the blue color of the stain. The number of viable cells were counted and calculated by using the formula below:

$$\text{No of cells} = \text{Counted cells} \times 5 \times (\text{dilution factor} = 2) \times 10^4 \times (\text{dilution factor} = 10)$$

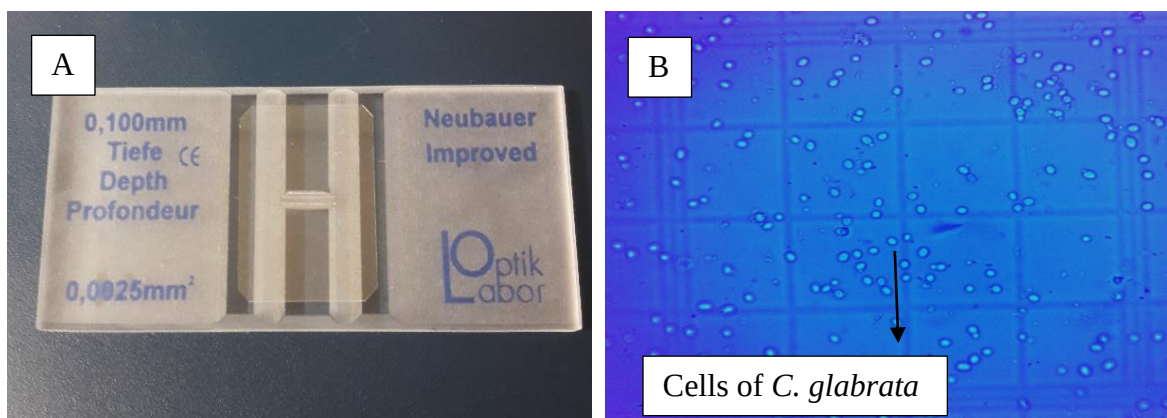


**Figure 3.12:** Cell count method (A) Haemocytometer; (B) *C. glabrata* yeast cells on haemocytometer, magnification 400x.

### 3.2.10.2 HPLC Analysis

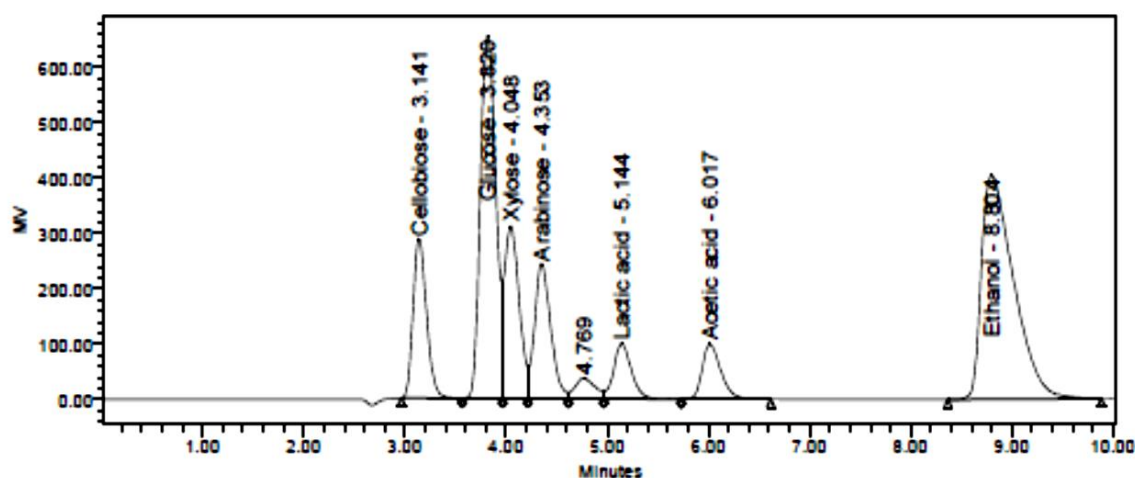
The supernatant collected were analysed using a High-Performance Liquid Chromatography (HPLC) (Waters 2695 Separations Module, Alliance HPLC System, United States) (Figure 3.13) for concentration quantification. All the fermentation products including ethanol, sugars and other fermentation by-products such as lactic acid and acetic acid were analysed according to standard protocols. The HPLC system used in this study was equipped with a column heater cooler (Alliance), refractive index detector (2414 RI Detector) and computer controller. The separation and analysis of ethanol and other fermentation constituents were done on a HPLC with conditions (Table 3.1) and the concentrations were determined based on a standard concentration curves according to retention times (Table 3.2) in chromatogram (Figure 3.14).



**Figure 3.13:** Main components of High-Performance Liquid Chromatography (HPLC), which consists of a pump, injector, column, detector and integrator and display system.

## SAMPLE INFORMATION

|                   |                          |                     |                       |
|-------------------|--------------------------|---------------------|-----------------------|
| Sample Name:      | standard 1               | Acquired By:        | System                |
| Sample Type:      | Standard                 | Sample Set Name:    | 20                    |
| Vial:             | 1                        | Acq. Method Set:    | Fern Lignocellulose 2 |
| Injection #:      | 1                        | Processing Method:  | STANDARD UPDATED      |
| Injection Volume: | 20.00 ul                 | Channel Name:       | 410                   |
| Run Time:         | 10.0 Minutes             | Proc. Chnl. Descr.: | 410                   |
| Date Acquired:    | 30/1/2019 5:06:51 PM MYT |                     |                       |
| Date Processed:   | 8/4/2019 2:44:05 PM MYT  |                     |                       |



|   | Peak Name   | RT    | Area    | % Area | Height | Amount  |
|---|-------------|-------|---------|--------|--------|---------|
| 1 | Cellobiose  | 3.141 | 2684982 | 9.79   | 290373 | 16.000  |
| 2 | Glucose     | 3.820 | 6968240 | 25.40  | 657248 | 48.000  |
| 3 | Xylose      | 4.048 | 3119313 | 11.37  | 312213 | 19.200  |
| 4 | Arabinose   | 4.353 | 2713373 | 9.89   | 244059 | 16.000  |
| 5 |             | 4.769 | 513347  | 1.87   | 37554  |         |
| 6 | Lactic acid | 5.144 | 1211472 | 4.42   | 100293 | 16.000  |
| 7 | Acetic acid | 6.017 | 1257940 | 4.59   | 99205  | 16.000  |
| 8 | Ethanol     | 8.804 | 8960595 | 32.67  | 405559 | 126.200 |

**Figure 3.14:** The chromatogram of High-Performance Liquid Chromatography, based on retention time.

**Table 3.1:** HPLC conditions (Vincent et al., 2011b).

| Parameter        | Conditions                                                     |
|------------------|----------------------------------------------------------------|
| Column           | Bio-Rad Aminex HPX-87H column (150 x 7.8 mm; Bio-Rad, CA, USA) |
| Mobile phase     | 0.005 M Sulphuric acid                                         |
| Flow rate        | 0.8 ml/min                                                     |
| Temperature      | 65 °C                                                          |
| Detector         | Refractive index                                               |
| Injection volume | 20 µL                                                          |

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

| Components  | Retention time (min) |
|-------------|----------------------|
| Maltose     | 3.188                |
| Cellobiose  | 3.141                |
| Glucose     | 3.820                |
| Sucrose     | 3.740                |
| Xylose      | 4.048                |
| Arabinose   | 4.353                |
| Lactic Acid | 5.144                |
| Acetic Acid | 6.017                |
| Ethanol     | 8.804                |

### 3.2.10.3 Phenol-sulfuric acid assay (PSA)

Determination of total carbohydrate was conducted via Phenol-sulfuric acid assay. The assay was performed by recording the optical density of the samples at 490 nm using a spectrophotometer (SP-880 Metertech, Taiwan). The amount of total carbohydrate present in the sample solution was calculated based on a standard curve (Appendix, Figure 2) that was plotted prior to the assay (Vincent et al., 2014).

### 3.2.11 Statistical analysis

All collected data from HPLC profiling were plotted into graphs on concentrations of glucose, maltose, sucrose, cellobiose, xylose, arabinose, lactic acid, acetic acid and ethanol. Cell growth of *C. glabrata* yeast cells and total carbohydrate content from PSA assay were also plotted into different graphs. Error bars were determined based on the standard deviation from the mean value of triplicate experiments. One-way analysis of variance (ANOVA) and Tukey's *post hoc* tests for significant differences were performed using IBM SPSS Statistics Software version 22 for all the final data set for comparison of ethanol yield of different substrates.

## CHAPTER 4

### RESULTS

#### 4.1 Evaluation of *Candida glabrata* cell growth

In the current study, ethanol production by *C. glabrata* using simple or complex carbohydrates as the sole carbon in a fermentation process was performed. The amount of sugar consumed by *C. glabrata* in each substrate was recorded. Ethanol yield was compared based on the theoretical value to determine the ability of *C. glabrata* as the fermenting organism in the fermentation process. The growth curves of *C. glabrata* in different sugar substrates are shown in Figure 4.1 and Figure 4.2. Figure 4.1 shows the pattern of cell growth over time in broths containing glucose, maltose and sucrose. At 0 h to 6 h, the cells were observed to be multiplying number in all broths. Subsequently, the cell number in the broth containing glucose showed a gradual increase that peaked at 48 h. After that, a gradual decline in cell concentration that continued until the end of the fermentation was observed. On the other hand, the broth containing maltose and sucrose showed a slower rate of cell growth compared to glucose. The data obtained showed that *C. glabrata* cells had a significantly higher total cell concentration in media containing 5% glucose than maltose and sucrose.

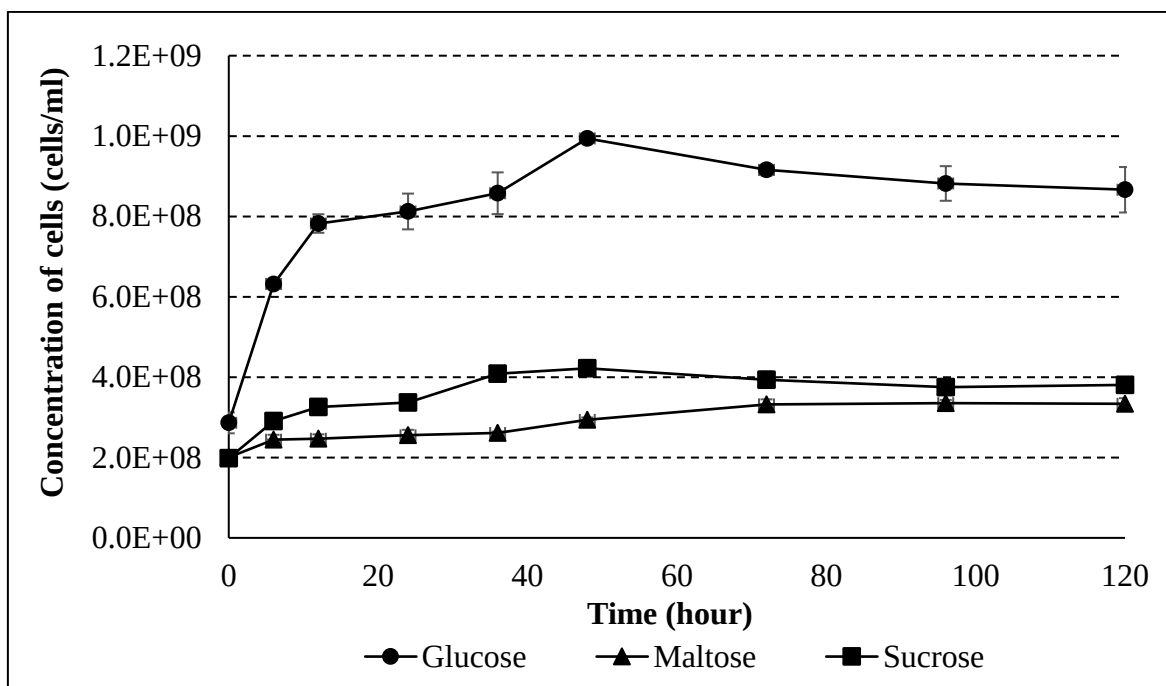


**Figure 4.1:** Time course of cell concentration in broths containing glucose, maltose and sucrose, as determined via cell count method. [Note: The data points represent the average of three independent experiments (n=3)]

The growth curves of *C. glabrata* in broths containing soluble starch and cellulose, with and without the addition of enzyme, are represented in Figure 4.2. For all conditions tested, a steady increase in cell concentration within the first 12 h was observed. The highest cell concentration was recorded in the broth containing starch with enzyme, followed by broth containing cellulose with enzyme. In the corresponding broths without the addition of enzymes, no significant difference in cell growth pattern was observed. A slow increase in the number of cells was noted throughout the fermentation period. Figure 4.3 shows the growth curve of *C. glabrata* throughout the SSF. The concentration of cells was observed to increase significantly within the first 6 h of SSF and remained constant until 96 h. After 96 h, the cell concentration decreased slowly until the end of the SSF.

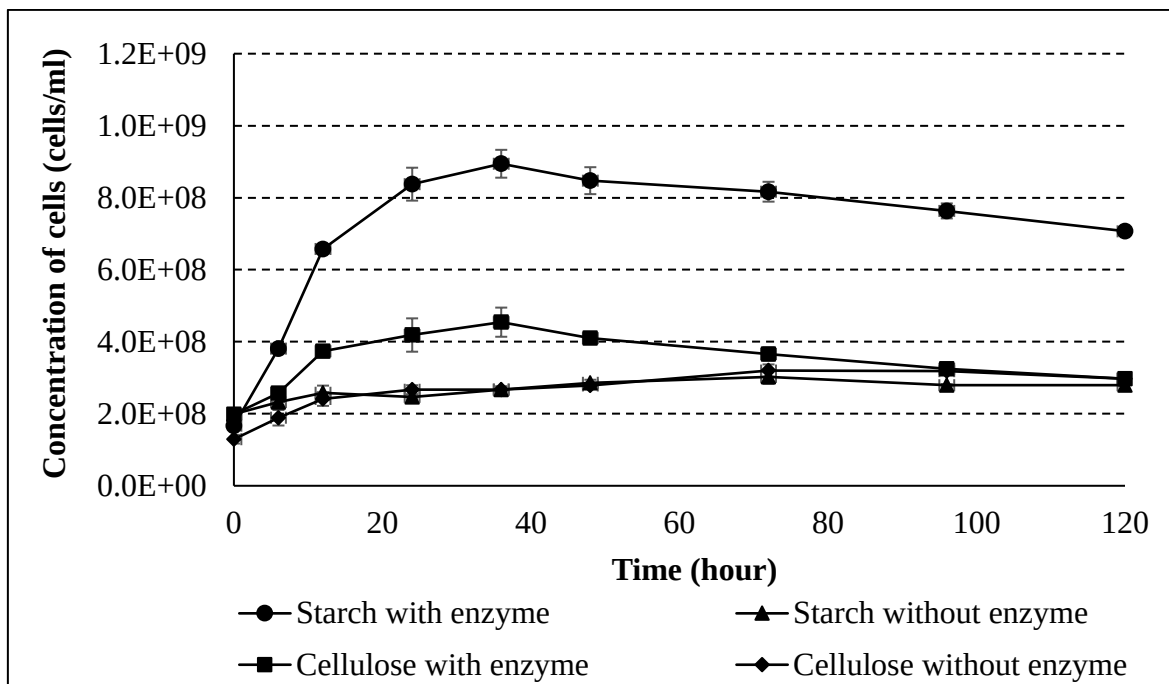


**Figure 4.2:** Time course of cell concentration in broths containing starch and cellulose (with or without enzyme), as determined via cell count method. [Note: The data points represent the average of three independent experiments (n=3)]

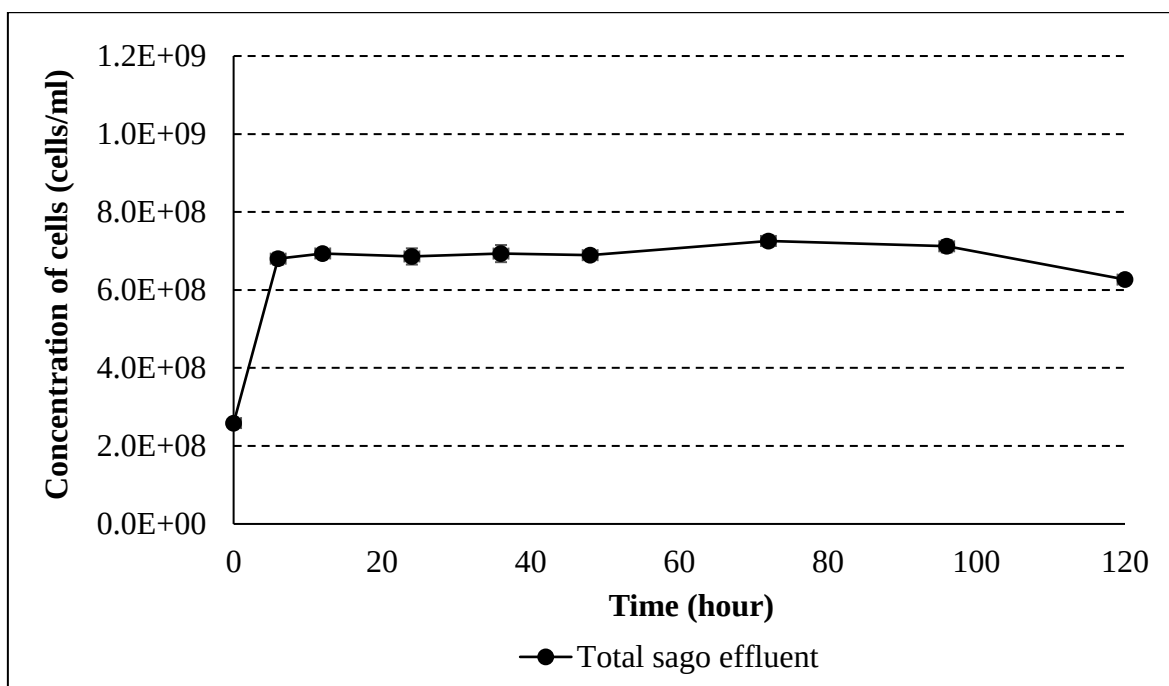


**Figure 4.3:** Time course of cell concentration in total sago effluent, as determined via cell count method. [Note: The data points represent the average of three independent experiments (n=3)]

## 4.2 Fermentation of glucose, maltose and sucrose

The concentration of ethanol, sugars and other fermentation by-products were determined by HPLC analysis. The concentration of ethanol produced in broths containing glucose, maltose and sucrose was shown in Figure 4.4. Ethanol yield is an important indicator in evaluating the ability of *C. glabrata* in producing ethanol using different types of substrate. Based on Figure 4.3, ethanol production in the broth containing glucose increased significantly from 0 h until 48 h. The highest ethanol yield was recorded at 72 h with a concentration of 20.40 g/l. After 72 h, the production of ethanol decreased slowly over time. On the other hand, no ethanol was detected throughout the 5 days of fermentation in the broth containing maltose. As for the broth containing sucrose, a low yield of ethanol was observed, with the highest peak of 1.64 g/l at 6 h.

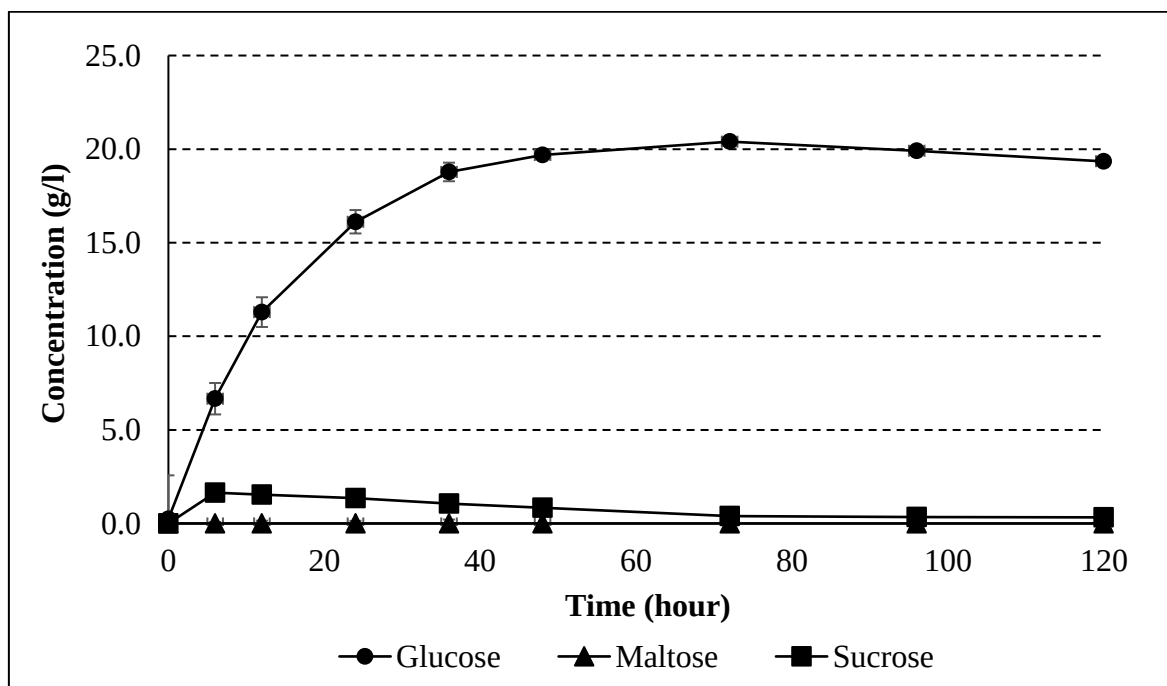


**Figure 4.4:** Time course of ethanol concentration in broths containing glucose, maltose and sucrose, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

Figure 4.5 illustrates the concentration of various sugars in the broths during the fermentation process. The initial concentration of glucose, maltose and sucrose were recorded in the range between 45.00 to 50.00 g/l. The concentration of glucose substantially reduced from the start of fermentation, from 46.44 to 8.31 g/l at 36 h. No glucose was detected in the broth after 48 h, up until the last sampling point of 120 h. However, the concentration of maltose and sucrose remained constant at 48.00 g/l throughout the fermentation period.

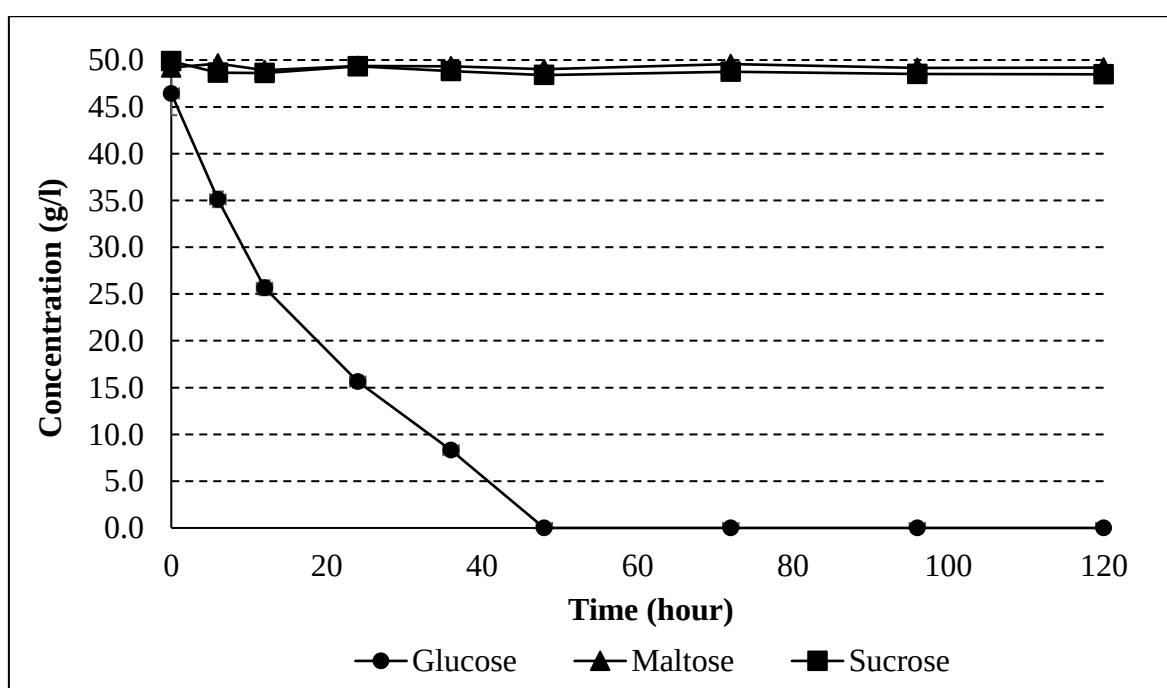


**Figure 4.5:** Time course of sugar concentration in broths containing glucose, maltose and sucrose, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

#### 4.3 Fermentation of soluble starch and cellulose powder

The ability of *C. glabrata* to produce ethanol from complex sugars with and without the addition of enzymes was further evaluated. Figure 4.6 and Figure 4.7 illustrate the production of ethanol and glucose in broths containing soluble starch, with and without the

addition of enzyme. As shown in Figure 4.6, ethanol production was recorded in the presence of enzyme, starting at 6 h and increased steeply for the next 12 h. A peak was observed at 24 h with a concentration of 15.48 g/l, after which the ethanol yield decreased slowly until the last sampling point. In contrast, no ethanol was produced in the absence of enzyme. The ethanol concentration was measured to be zero from the start to the end of the fermentation. Figure 4.7 shows the glucose concentration throughout the fermentation process, where the initial concentration of 53.69 g/l was recorded. In the next 6 h, the glucose concentration sharply reduced to 11.71 g/l and this continued until 12 h. From there onwards, no more glucose was detected in the broth. On the other hand, no glucose was detected from 0 to 120 h of fermentation in the absence of enzyme.

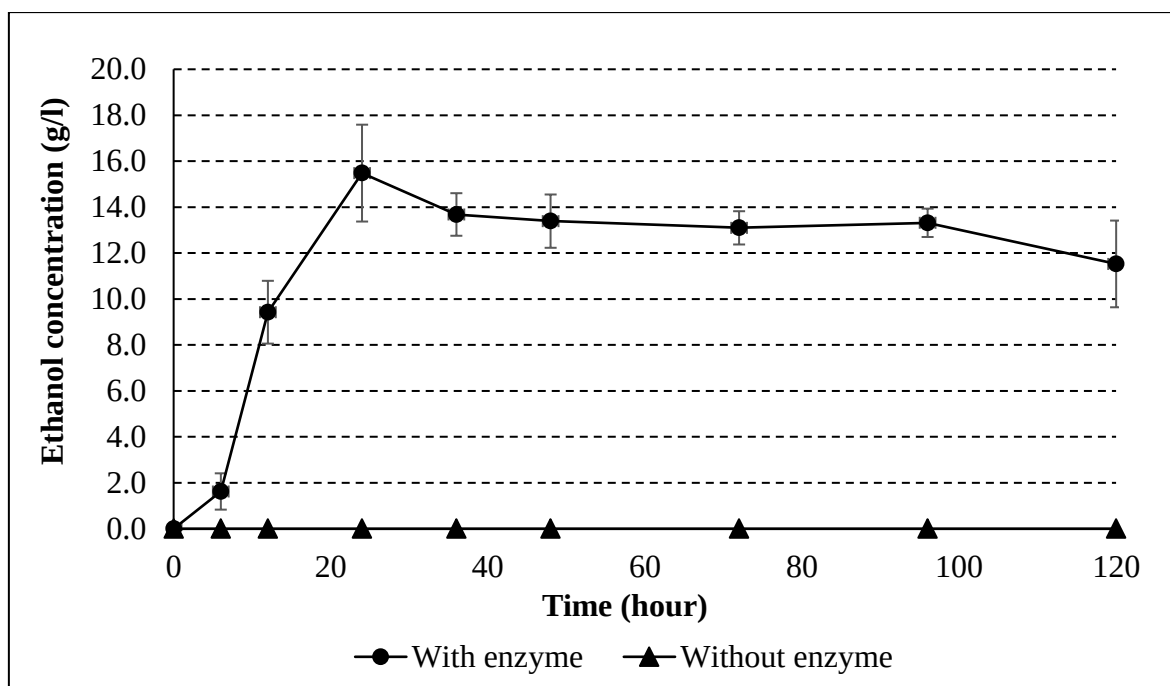


**Figure 4.6:** Time course of ethanol concentration in broths containing soluble starch (with and without the addition of enzyme), as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

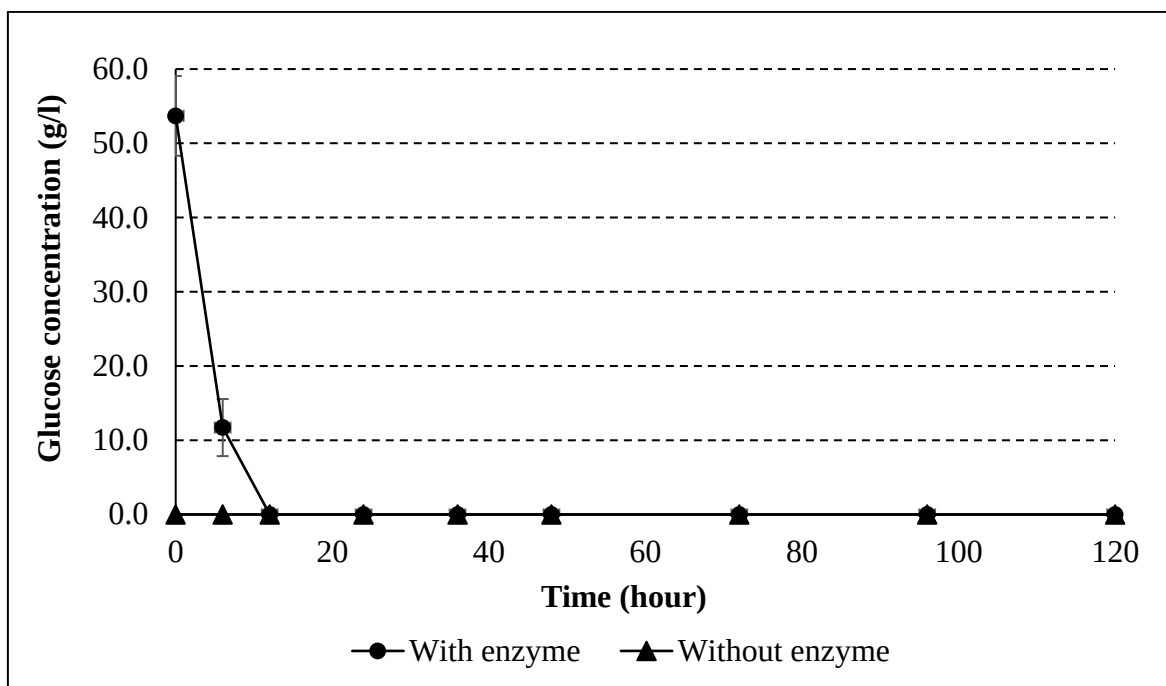


**Figure 4.7:** Time course of sugar concentration in broths containing soluble starch (with and without the addition of enzyme), as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

The concentrations of ethanol and glucose produced in broths containing cellulose powder are represented in Figure 4.8 and Figure 4.9, respectively. Based on the data results, ethanol yield was only observed in the broth containing the enzymes. The concentration of ethanol increased gradually over the 36 hours of fermentation, with highest production about 8.83 g/l. After that, the ethanol yield slowed down slightly and remained constant until the end of the fermentation. As shown in Figure 4.9, the initial glucose concentration was recorded at 1.05 g/l and the glucose level was observed to decline over time. At 24 h onwards, no more glucose was detected in the broth. On the contrary, in the absence of enzymes, no ethanol yield was measured throughout the fermentation, as shown in Figure 4.8. Similarly, the glucose concentration remained to be zero from the beginning until the end of fermentation (Figure 4.9).

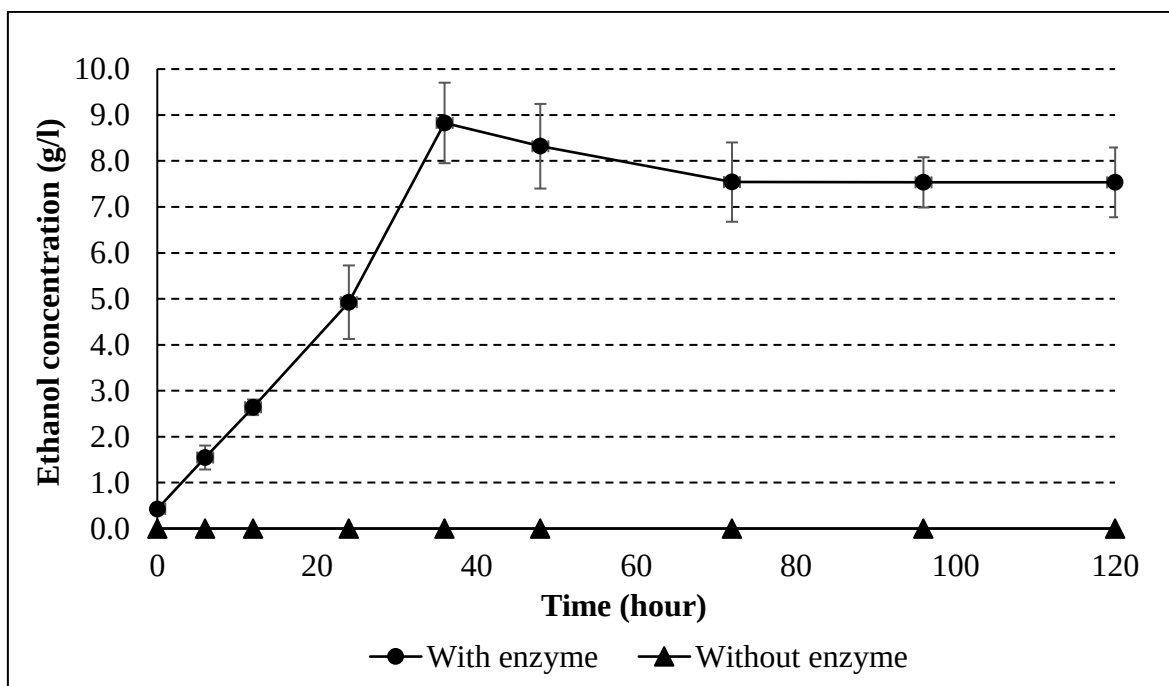


**Figure 4.8:** Time course of ethanol concentration in broths containing cellulose (with and without the addition of enzyme), as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

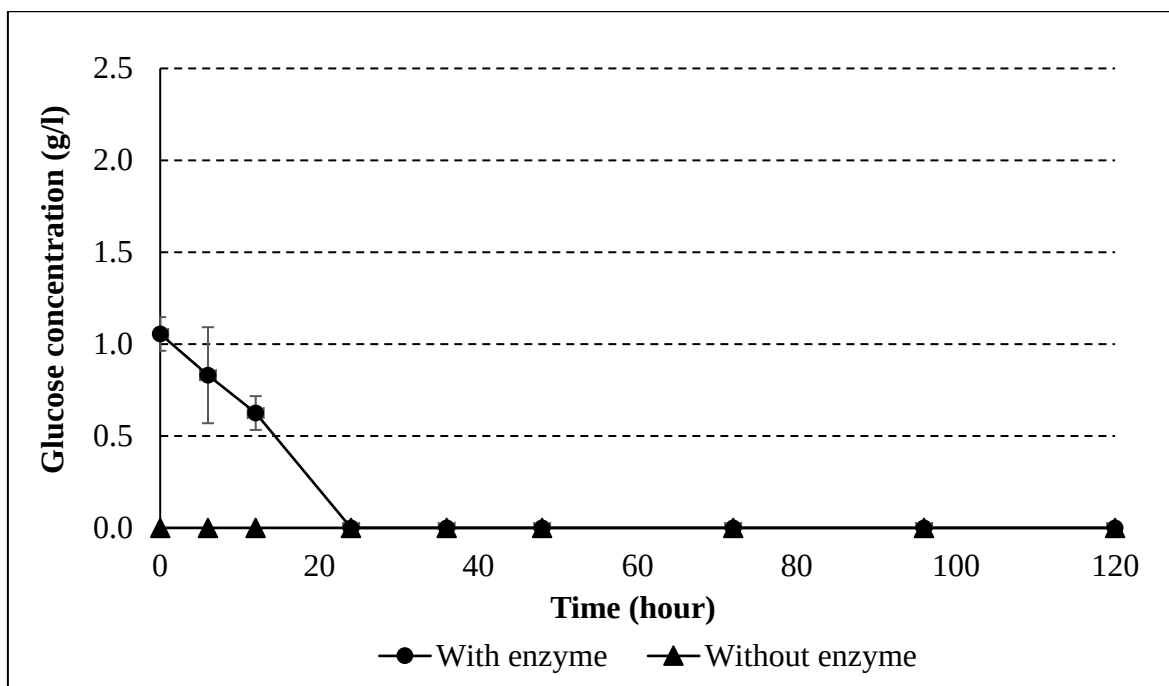


**Figure 4.9:** Time course of sugar concentration in broths containing cellulose (with and without the addition of enzyme), as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

Production of other sugar in the broth containing cellulose powder such as cellobiose also being quantified and the graph of the concentration was shown in Figure 4.10 below. Based on the graph, the initial concentration of cellobiose was 3.73 g/l. After that, the concentration was increased until maximum production at 12 h, with concentration of 6.30 g/l. Then, the concentration was decreased steadily in the next following hours. At 36 h and onwards, cellobiose was no longer detected in the broth.

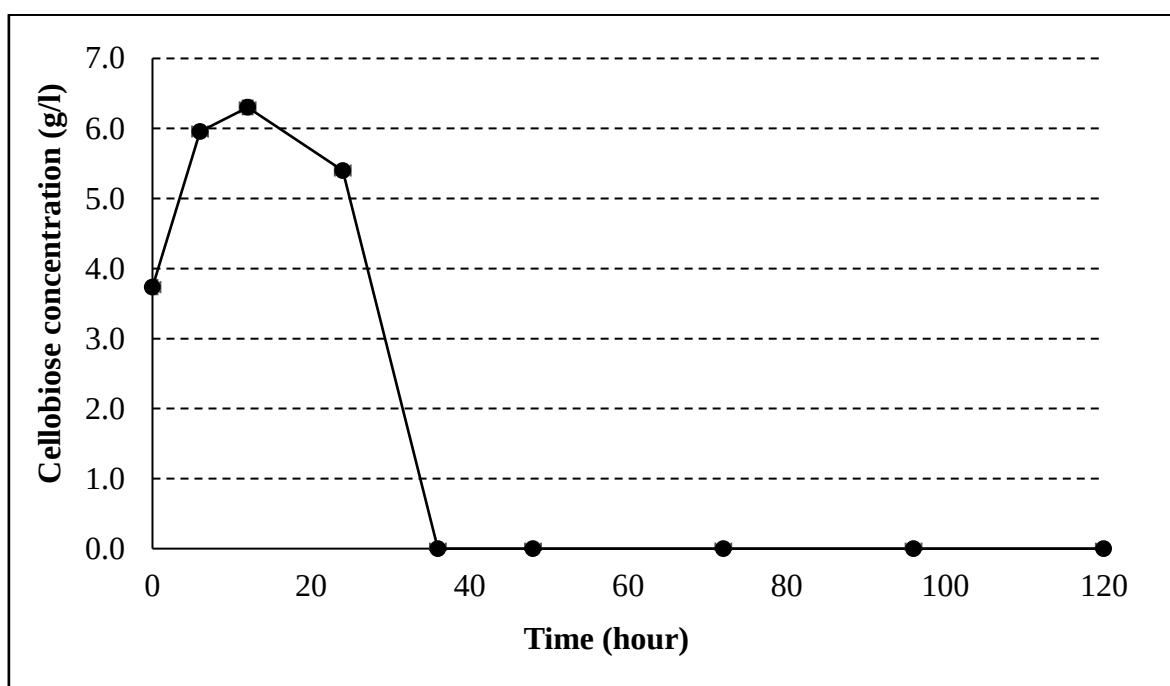


**Figure 4.10:** Time course of cellobiose concentration in broth containing cellulose powder with enzyme, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

Other fermentation components that were quantified using HPLC analysis were lactic acid and acetic acid. As shown in Figure 4.11, the broth containing glucose showed a higher production of lactic acid, compared to the other sugars. A sharp increase from 0.36 to 3.52 g/l was recorded for the first 12 h, followed by a peak of 3.71 g/l at 24 h. In broths containing maltose and sucrose, a very low amount of lactic acid were detected in the early stage of fermentation. After 24 h, there was almost no lactic acid was detected.

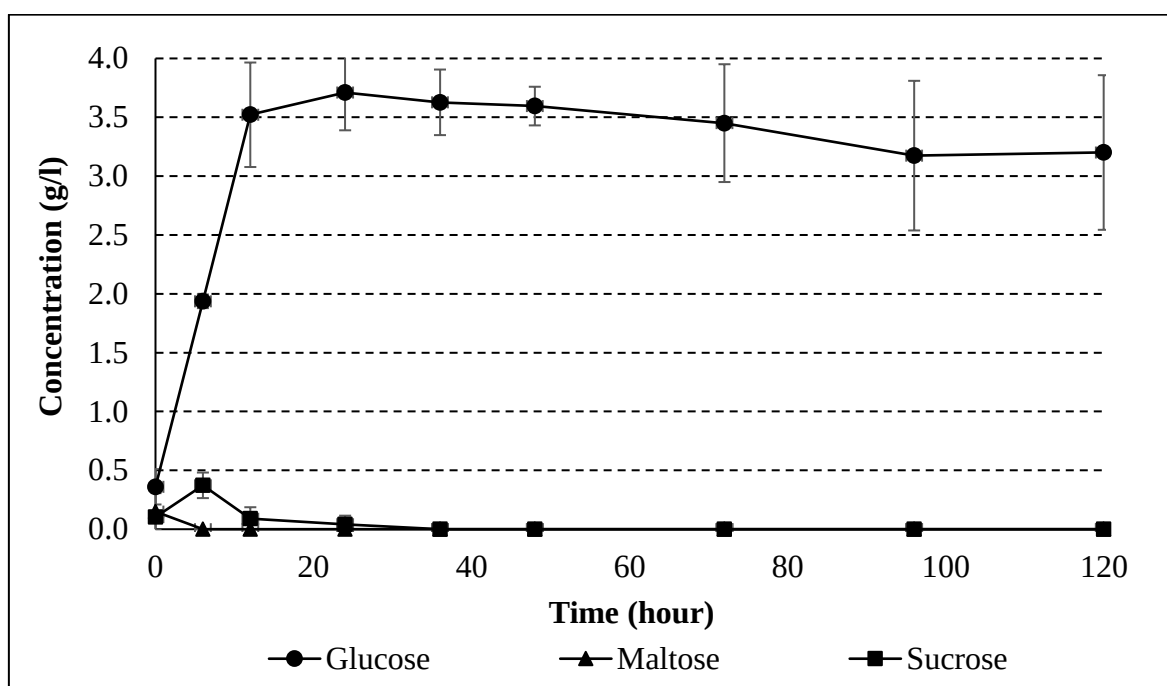


**Figure 4.11:** Time course of lactic acid concentration in broths containing glucose, maltose and sucrose, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

Figure 4.12 illustrates the concentration of acetic acid present in the fermentation system. Overall, the level of acetic acid in all broths was recorded to be lower than 1.00 g/l and no acetic acid was detected at the beginning of the fermentation. After 24 h, the concentration increased slightly to 0.34 g/l in the broth containing glucose and remained constant until the end of the fermentation. Meanwhile, a very small amount of acetic acid was detected in the

broth containing sucrose, with 0.14 g/l at 48 h. After 72 h, no more changes in acetic acid concentration was observed.

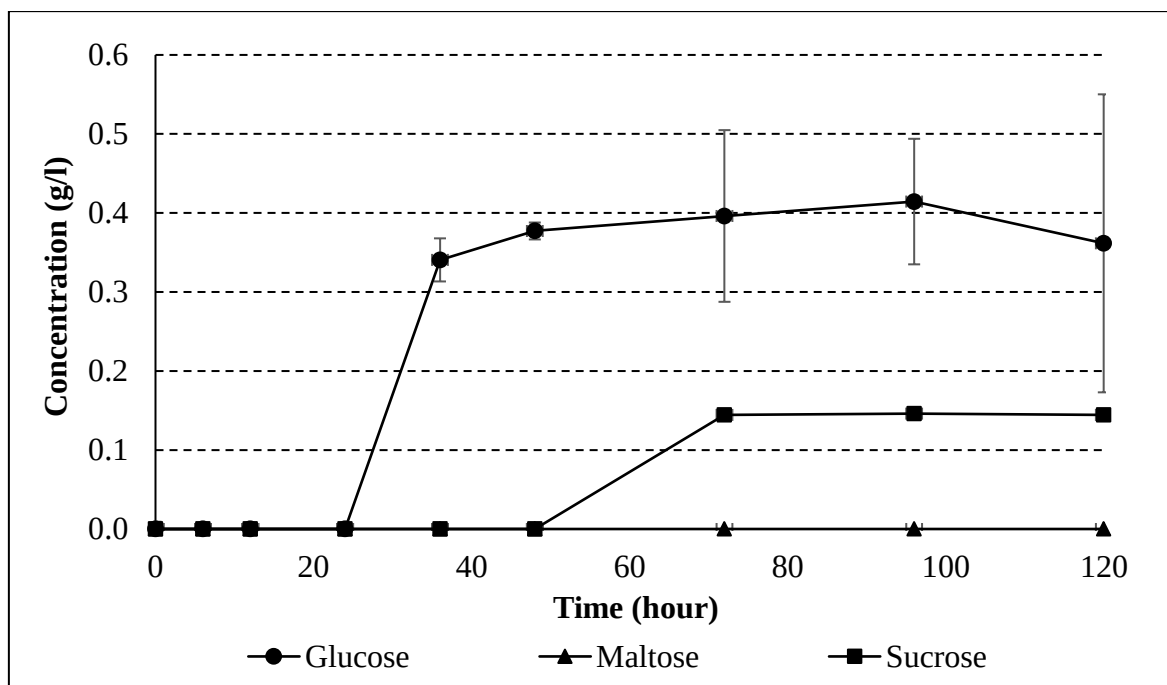


**Figure 4.12:** Time course of acetic acid concentration in broths containing glucose, maltose and sucrose, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

The concentration of lactic acid in broths containing starch or cellulose, with and without the addition of enzyme is shown in Figure 4.13. The lactic acid concentration in the broth containing starch with enzyme was found to increase steadily from 0 to 0.2 g/l within the first 24 h. Subsequently, the concentration declined gradually over time until no more lactic acid was being detected from 96 h onwards. In the meantime, lactic acid was found to be present in the broth containing cellulose with enzyme during the first 12 h, with an initial concentration of 0.2 g/l. After 12 h, the concentration has dropped to zero and remained the same until the end of the fermentation. In contrast, the other two broths without enzymes showed no production of lactic acid throughout the fermentation period.

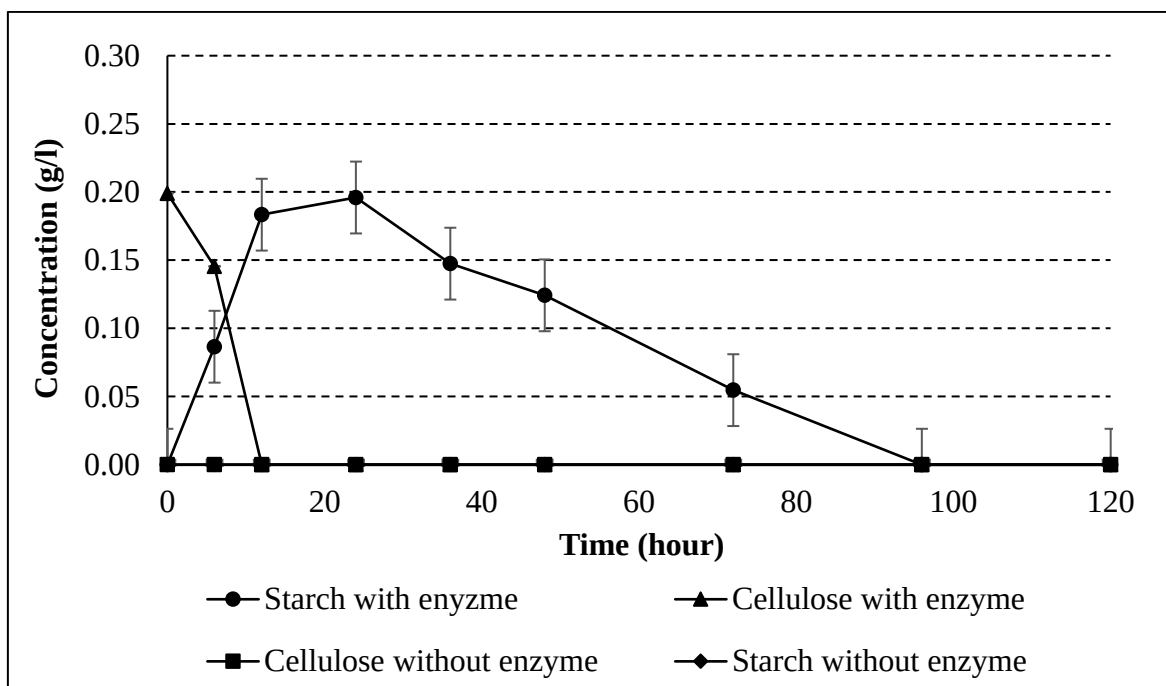


**Figure 4.13:** Time course of lactic acid concentration in broths containing starch or cellulose (with and without the addition of enzyme), as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

Figure 4.14 illustrates the concentration of acetic acid in the same fermentation conditions. The presence of acetic acid was only detected after 72 h in the broth containing starch with the enzymes. The peak was recorded at 120 h with a concentration of 0.15 g/l. On the contrary, in broth containing cellulose with the enzymes, acetic acid production was observed in the early stage of fermentation. The initial acetic acid concentration of 0.27 g/l dropped rapidly to zero in 6 hours. No detection of acetic acid was made in the broths without enzyme throughout the fermentation period.

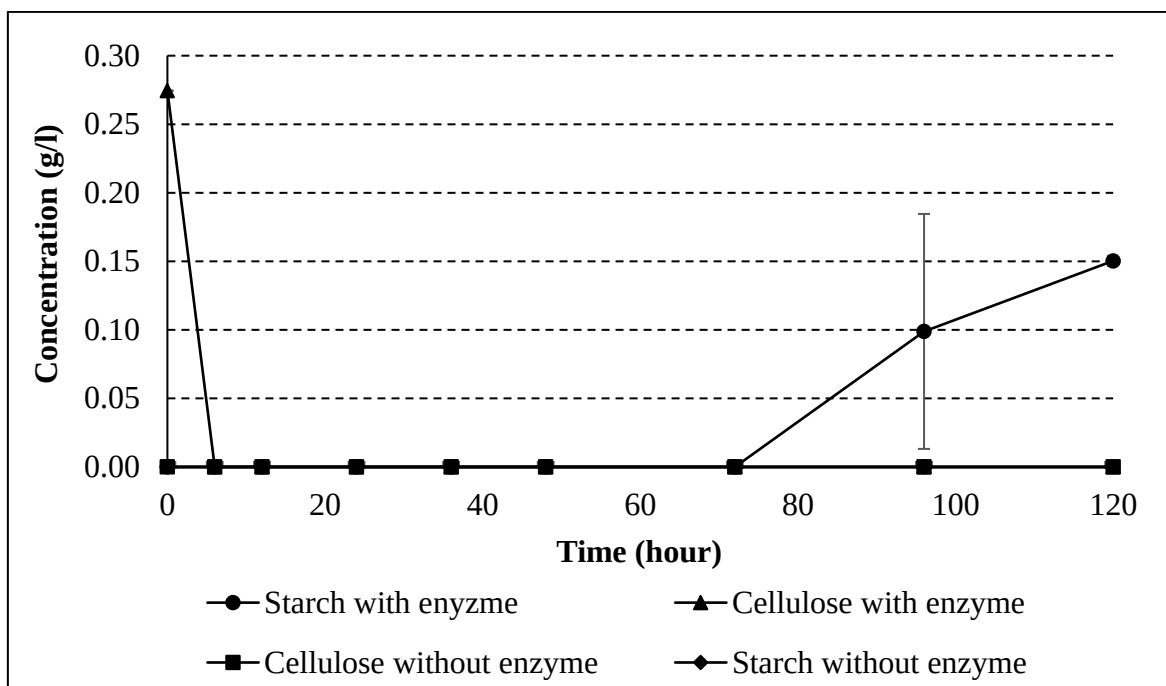


**Figure 4.14:** Time course of acetic acid concentration in broths containing starch or cellulose (with and without the addition of enzyme), as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

#### 4.4 Simultaneous saccharification and fermentation (SSF) of total sago effluent

In this study, the production of bioethanol via SSF was conducted at a working volume of 150 ml of total sago effluent. Composition analyses were done prior to the SSF process and the data obtained are presented in Table 4.1 and Table 4.2. Table 4.1 shows the compositions of total sago effluent, which comprises  $97.14 \pm 0.09\%$  sago hydrolysate and  $2.86 \pm 0.09\%$  solid component (sago hampas). The main components of dried sago hampas are summarized in Table 4.2. In summary, the components are  $52.03 \pm 1.11\%$  starch,  $27.82 \pm 0.62\%$  cellulose,  $5.32 \pm 0.96\%$  hemicellulose,  $3.00 \pm 0.05\%$  lignin and  $1.95 \pm 0.03\%$  ash in dry basis (w/w).

**Table 4.1:** Compositional analysis of total sago effluent.

| Compositions        |                 | Amount (%)    |              |
|---------------------|-----------------|---------------|--------------|
| Total Sago Effluent | Sago effluent   | Water         | 94.91 ± 0.09 |
|                     | hydrolysate     | Free Starch   | 2.23 ± 0.86  |
|                     | Dry sago hampas | Starch        | 1.49 ± 1.11  |
|                     |                 | Cellulose     | 0.79 ± 1.23  |
|                     |                 | Hemicellulose | 0.15 ± 2.00  |
|                     |                 | Lignin        | 0.09 ± 0.09  |
|                     |                 | Ash           | 0.06 ± 0.06  |
|                     |                 | Others        | 0.28 ± 0.00  |

**Table 4.2:** Composition analysis of dried sago hampas.

| <b>Compositions</b> | <b>% w/w</b> |
|---------------------|--------------|
| Starch              | 52.03 ± 1.11 |
| Cellulose           | 27.82 ± 0.62 |
| Hemicellulose       | 5.32 ± 0.96  |
| Lignin              | 3.00 ± 0.05  |
| Ash                 | 1.95 ± 0.03  |
| Others              | 9.88 ± 0.07  |

The SSF of total sago effluent was conducted for 5 days and samplings were done at 0, 6, 12, 24, 36, 48, 72, 96, 120 h. From the collected samples, phenol-sulfuric acid assay and HPLC profiling were performed. In this study, the total carbohydrate concentration was determined by using the phenol-sulfuric acid assay. The results were presented in Figure 4.15. The initial total carbohydrate concentration in the fermentation broth was 20.57 g/l and is the highest compared to other sampling hours. At 6 h, the total carbohydrate content has declined steeply from 20.57 to 4.98 g/l, which is a total reduction of approximately 75%. At 12 h, the concentration decreased further to 4.02 g/l and remained relatively constant until the end of fermentation.

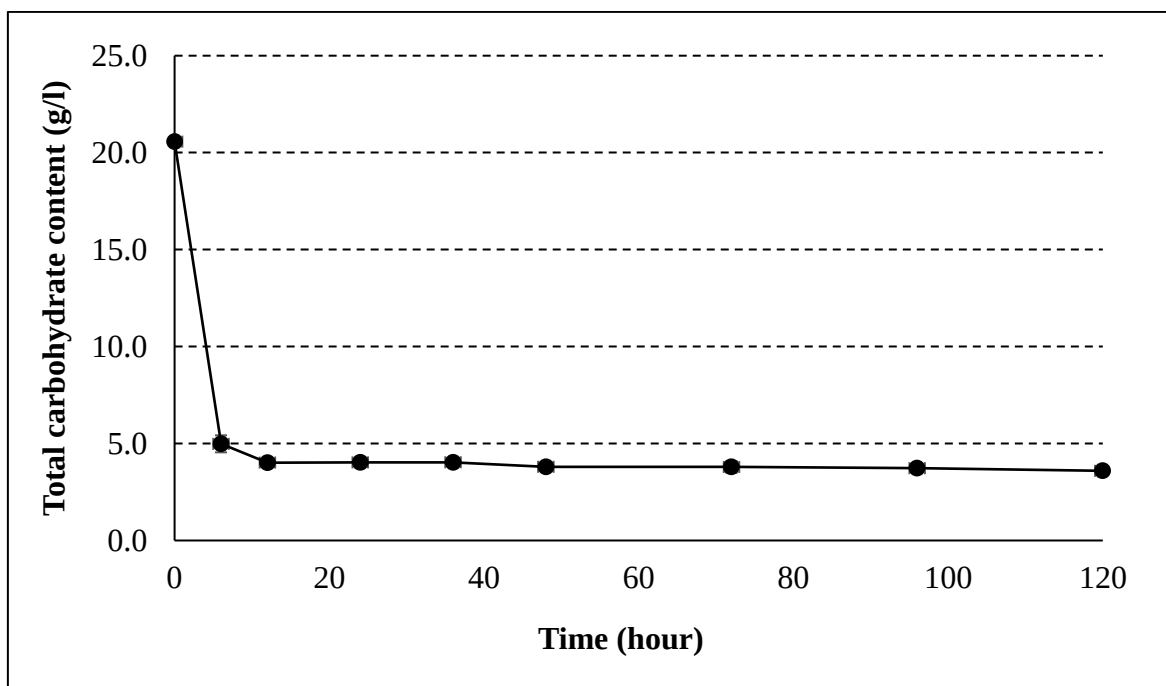


**Figure 4.15:** Time course of total carbohydrate content in total sago effluent, as determined via the phenol-sulfuric acid assay. [Note: The data points represent the average of three independent experiments (n=3)]

The concentration of ethanol, glucose, cellobiose, xylose, arabinose, lactic acid and acetic acid produced were determined by HPLC analysis. The time course of each component was shown in Figure 4.16 to Figure 4.18. Figure 4.16 illustrates the concentration of glucose and ethanol produced in TSE broth during SSF. The initial concentration of glucose was 16.47 g/l at 0 h. Then, the concentration was observed to decrease rapidly to 0.56 g/l in the first 6 hours. At 12 h onwards, no more glucose was detectable. On the contrary, the production of ethanol was found to increase significantly from 0 to 12 h, with the concentration of zero to 4.21 g/l. The peak was observed at 24 h, with the ethanol concentration of 5.44 g/l. Subsequently, the ethanol concentration declined slowly until the end of the SSF.

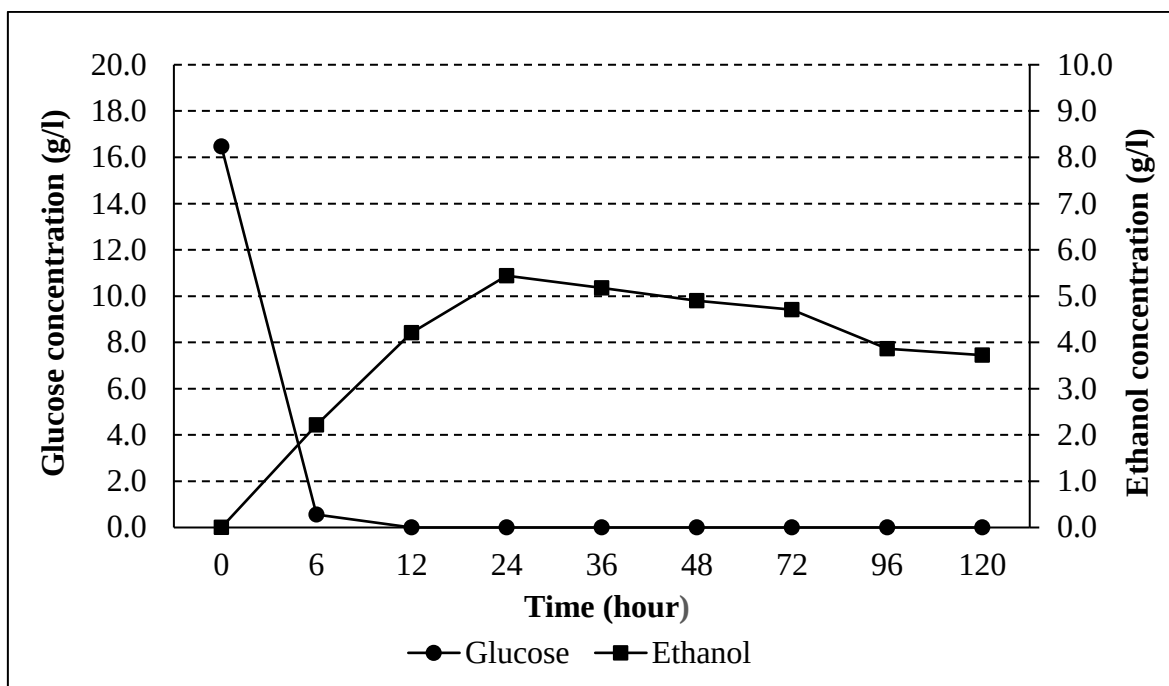


**Figure 4.16:** Time course of glucose concentration and ethanol concentration in total sago effluent, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

Figure 4.17 shows the concentration of cellobiose, xylose and arabinose during the SSF process. The initial concentration of cellobiose was 4.32 g/l at 0 h, followed by a slight increase to 4.52 g/l at 6 h. After that, the concentration was decreased to 2.39 and 1.62 g/l in the next sampling hours. Then, the concentration was observed to be constant until the last sampling hour. On the contrary, there was no xylose was detected at the initial stage of SSF, up until 12 h when a concentration of 0.19 g/l was measured. The xylose concentration increased slightly to 0.27 g/l at 24 h and remained constant at 0.25 g/l until the end of the SSF. Arabinose was also detected at the beginning of SSF, with a concentration of 0.40 g/l. The concentration rose gradually to 0.80 g/l at 12 h. At 24 h onward, the concentration fluctuated slightly between 0.8 and 0.9 g/l, before peaking to 1.10 g/l at 120 h.

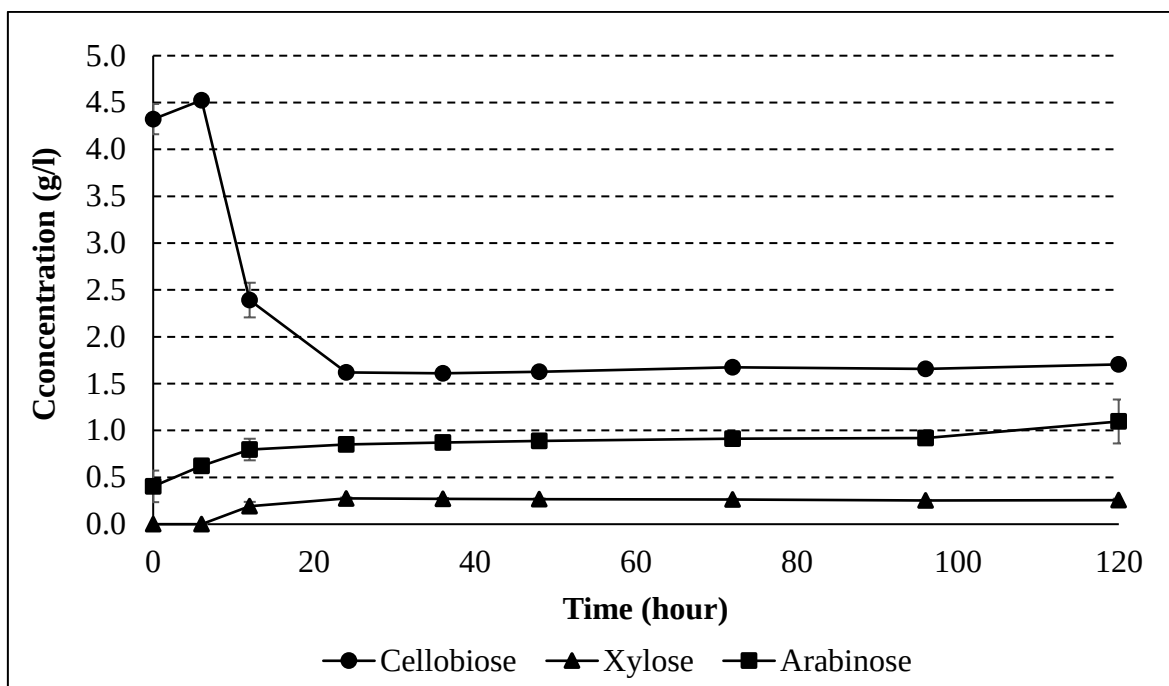


**Figure 4.17:** Time course of cellobiose, xylose and arabinose concentration in total sago effluent, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

Other fermentation by-products such as lactic acid and acetic acid were quantified using HPLC. Figure 4.18 shows the concentration of lactic acid that remained relatively constant between 0.1 to 0.2 g/l throughout the SSF. Similarly, there was no significant change in acetic acid level throughout the SSF, as shown in Figure 4.18. The initial acetic acid concentration was recorded at 0.46 g/l and fluctuated slightly between 0.2 to 0.4 g/l from 6 h until the end of SSF.

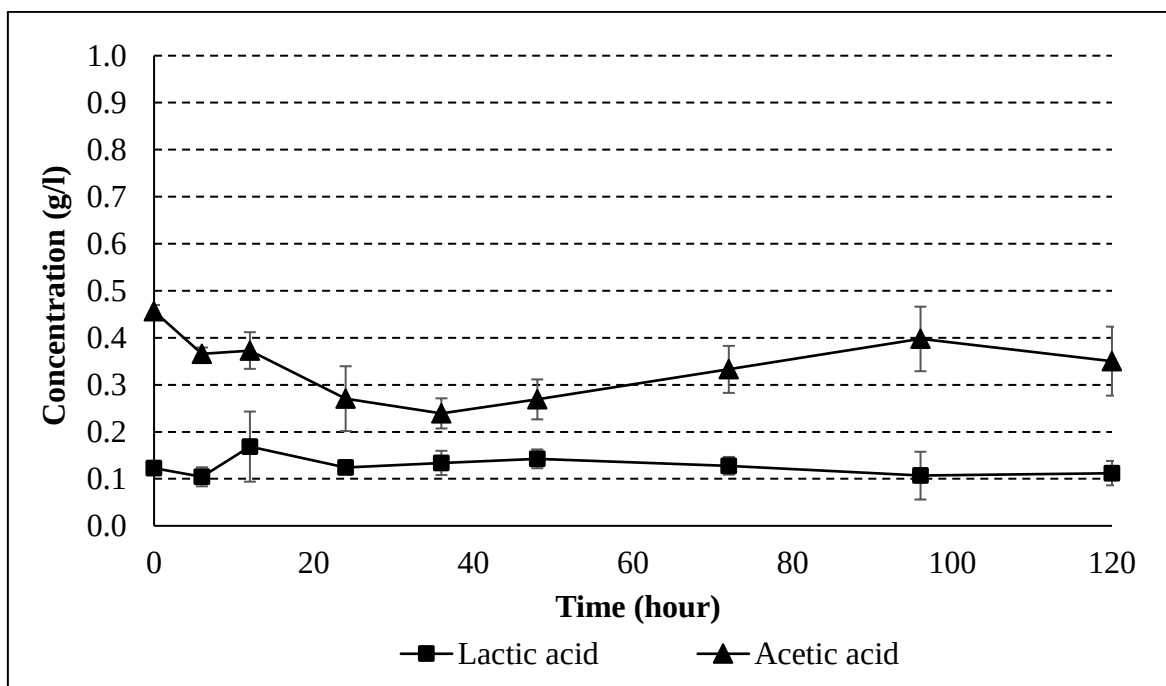


**Figure 4.18:** Time course of lactic acid and acetic acid concentration in total sago effluent, as determined via HPLC. [Note: The data points represent the average of three independent experiments (n=3)]

#### 4.5 Evaluation of ethanol-producing ability of *C. glabrata*

Table 4.3 depicts the overall maximum ethanol production and yield from the substrates tested in this study. Among simple and complex sugars, the highest ethanol production was recorded in the broth containing glucose. A total of 20.4 g/l of ethanol was produced, corresponding to a theoretical yield of 79.84%. Starch with enzyme was found to be the second highest ethanol producer, with a concentration of 15.23 g/l and a theoretical yield of 55.08%. The theoretical ethanol yield for broths containing sucrose and cellulose with the enzymes are 6.44% and 31.41%, respectively. In contrast, there was no ethanol yield in broths containing maltose, starch and cellulose without enzymes. Among the various substrates tested, only glucose and sucrose were consumed directly by *C. glabrata* to produce ethanol. Meanwhile, it is observed that soluble starch and cellulose powder were

consumed only with the addition of enzymes, with the former giving a higher yield. The data obtained also showed that the percentage of ethanol yield from glucose (79.84%) was the highest among the substrates. On the other hand, the maximum ethanol production by total sago effluent was recorded at 5.54 g/l with a percentage yield of 21.25%. Further statistical analysis was conducted using Tukey *pos hoc* test (multiple comparisons). Figure 4.19 indicates the statistically significant experimental theoretical ethanol yield among the different types of substrates, with p-value < 0.05. The overall processed data are shown in the Appendices.

**Table 4.3:** Maximum production and ethanol yield of each substrate.

| Substrate                | Maximum production of ethanol (g/l) | Experimental theoretical yield (%) |
|--------------------------|-------------------------------------|------------------------------------|
| Glucose                  | 20.40 ± 0.30                        | 79.84 ± 1.18 <sup>a</sup>          |
| Maltose                  | 0.00 ± 0.00                         | 0.00 ± 0.00 <sup>b</sup>           |
| Sucrose                  | 1.64 ± 0.08                         | 6.44 ± 0.30 <sup>b</sup>           |
| Starch with enzyme       | 15.23 ± 2.11                        | 55.08 ± 7.50 <sup>d</sup>          |
| Starch without enzyme    | 0.00 ± 0.00                         | 0.00 ± 0.00 <sup>b</sup>           |
| Cellulose with enzyme    | 8.83 ± 0.76                         | 31.41 ± 3.31 <sup>e</sup>          |
| Cellulose without enzyme | 0.00 ± 0.09                         | 0.00 ± 0.00 <sup>b</sup>           |
| Total sago effluent      | 5.44 ± 0.09                         | 21.25 ± 0.36 <sup>e</sup>          |

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

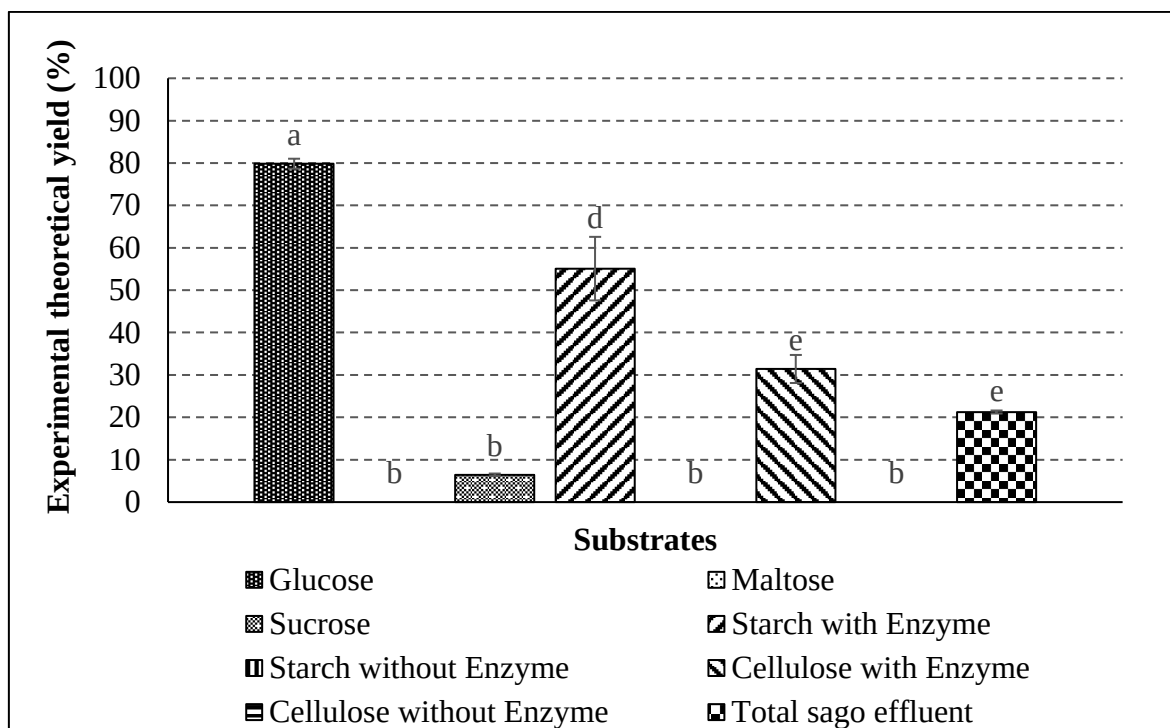


**Figure 4.19:** Experimental theoretical ethanol yield by different types of substrates. [Note: Different letters on top of column indicate statistically significant differences via Tukey's *post hoc* test,  $\alpha=0.05$ ]

## CHAPTER 5

### DISCUSSION

Ethanol is one of the fermentation products by yeast with sugar as the carbon source. Essentially, the fermentation of different plant carbohydrates using yeast is one of the oldest human technologies. Yeasts are indispensable for many biotechnological processes including the production of beer, wine and biofuel (Sicard & Legras, 2010; Dashko et al., 2014). Production of bioethanol from biomass with different yeast strains and fermentation conditions could result in different ethanol yields. High production of bioethanol is crucial to fulfill the increasing worldwide demand (Azhar et al., 2017). Multiple of studies have been conducted in the past years to enhance the production of bioethanol, particularly targeting biomass fractionation processes that are more economic and viable (Sun & Cheng, 2002; Carvalheiro et al., 2008), in addition to a multiproduct biorefinery conceptualization for process and product economics (Ghosh et al., 2015).

Various microorganisms have been tested for their fermentation capacity, including yeast, fungi, and bacteria. The most common microorganism used is *Saccharomyces cerevisiae* due to its high efficiency in converting sugar to alcohol (Kosaric & Velikonja, 1995). In order to improve sugar utilization and ethanol production, a genetic engineering approach to yeast fermentation has been thoroughly studied (Ha et al., 2011; Liu et al., 2011; Carrasco et al., 2013; Pagliardini et al., 2013). Recent interests have also been drawn to the search for new fermenting yeasts that may perform better than *Saccharomyces* (Nonklang et al., 2008).

Yeasts are commonly known to have a broad range of carbon sources that can support their growth, although they prefer to metabolize simple sugars. Sugars are classified into monosaccharides, disaccharides and polysaccharides. Monosaccharides are the preferred carbon sources for the yeasts in fermentation process (Battaglia et al., 2011). According to Ruriani et al. (2012), yeast cells basically use monosaccharide for their growth, but only few of the monosaccharide compounds such as glucose, galactose and mannose can be converted into ethanol. Pentose sugars (five-carbon sugars) such as xylose and arabinose, can only be fermented by a small number of wild microorganisms, often with a low ethanol yield. (Mosier, 2005; Hisamatsu et al., 2006). In general, yeasts break down the sugar into alcohol and carbon dioxide during fermentation. Microorganisms that are involved in fermentations have the ability to utilize glucose in the absence of oxygen to produce energy, ethanol and carbon dioxide (Ingram et al., 1998; Deesuth et al., 2012). These characteristics are crucial for them to be targeted as potential bioagents in fermentation technology (Zabed et al., 2014).

Yeast strains differ widely in their ability to produce ethanol. In the current study, *Candida glabrata* was chosen as the fermenting agent. The ability of *C. glabrata* in fermenting sugars was studied based on the ethanol yield at the end of the fermentation. The yield depends on multiple factors such as yeast strain, growth factors and fermentation conditions (Khongsay et al., 2010). According to Walker and Stewart (2016), ethanol is produced as cells strive to maintain their redox balance and yield ATP for continued growth. A rapid and efficient fermentation is crucial to yield high ethanol concentrations therefore the selected yeast strain must possess a good specific growth rate (Chang et al., 2018). For this reason, the cell growth of *C. glabrata* was studied to determine its growth profile in glucose, maltose and sucrose.

Based on the results, the cells were in the growth phase during the first 12 h in all types of media tested. The cell concentration in the cultures was observed to increase rapidly in this phase. This indicates that the cells were actively dividing and growing in number. During fermentation, the period of maximum sugar uptake and alcohol production was reported to coincide with the logarithmic phase. On top of that, the growing cells were estimated to produce ethanol 33 times faster than non-growing cells (Ingledew, 1999). After a specific period, the cells started going into the stationary phase where the carbon sources have been fully consumed. Eventually the cells decreased in number due to the ethanol toxicity and lack of nutrition (Formakis & Koliadima, 2005; Farmakis et al., 2007; Lainioti et al., 2010). Alcohol accumulation is known to inhibit yeast growth and viability (Jönsson et al., 2013). In the current study, the cells were observed to either maintain or decrease in number at the last sampling hour.

In the first part of this study, simple sugars (glucose, maltose and sucrose) were used as the substrate in the fermentation system and the yield of ethanol was determined via HPLC analysis. Based on the results obtained, *C. glabrata* consumed glucose and sucrose with the maximum ethanol production of approximately 20.4 g/l and 1.64 g/l, respectively. This was supported by a previous report by Barnett et al. (2000), on the ability of *C. glabrata* to convert sugar into ethanol. Although the carbon utilization range of *C. glabrata* is extremely narrow when compared with other yeasts (Hittinger et al., 2004; Kaur et al., 2005; Roetzer et al., 2011; Brunke & Hube, 2013; Turner & Butler, 2014), *C. glabrata* was reported to be capable of consuming two types of sugars, glucose and trehalose, a disaccharide (Barnett et al., 2000). In this study, ethanol was detected in the fermentation broth containing sucrose, at a very low concentration of 6.44% of the theoretical value. In the meantime, the ethanol

yield from glucose was measured to be 79.84% of the theoretical value, indicating that *C. glabrata* was capable of fermenting glucose efficiently among simple sugars. A similar study was conducted by Techaparin et al. (2017), where *C. glabrata* was found to ferment glucose and produced approximately 40.0 g/l of ethanol or 42% of the theoretical yield. By comparison, our study showed a higher efficiency of ethanol production, which could be the result of a different strain being used. Other conditions such as temperature and initial concentration of substrates could also play a role.

As presented in Figure 4.19, only the broth containing glucose showed a significant yield while the others did not. According to Mosier (2005), hexoses such as glucose can be fermented by most species of microorganism especially yeast, resulting in a high production of ethanol when compared to other substrates. Furthermore, yeast cells are found to grow optimally in simple sugar such as glucose (Chavan et al., 2009; Ocon et al., 2010). D-Glucose is also considered as the best substrate for the growth of yeast cells and ethanol fermentation (Ruriani et al., 2012). Lagunas (1993) reported that glucose has the fastest rate of carbon dioxide production when compared to other sugars. This indicates that in any fermentation, glucose is rapidly metabolized by the microorganisms to produce ethanol, which occurs even in the early stage of fermentation. The findings in our study highly correlates to this phenomenon, as the production of ethanol was detected up to 6.0 g/l in the first 6 h of fermentation.

At the initial stage of fermentation, oxygen is needed by the yeast cells for growth. However, the reaction eventually becomes anaerobic with the accumulation of carbon dioxide, where

the cells actively consume the carbon source such as glucose (Ruriani et al., 2012). During anaerobic growth, yeast cells basically metabolize glucose into ethanol through the reaction known as Embden Meyerhoff Parnas (EMP) Pathway, in which each mole of glucose generates two moles of ethanol, carbon dioxide and ATP. Theoretically, each gram of glucose will yield 0.51 g of ethanol. From the findings of this study, glucose produced ethanol to about 79.84% of theoretical value and this is considered as a good yield. The ethanol yield is usually lower than 90% and is limited by the nutrients or substrate used to synthesize biomass and maintain the reactions during fermentation. Therefore, the amount of ethanol generated in this study is not practical for industrial application, as the yield should constitute as much as 93% of its theoretical value of the ratio of ethanol produced to sugar consumed (Ingledew, 1999).

Further evaluations were conducted using polysaccharides such as starch and cellulose as substrate. Starch is the second most abundant carbohydrate in the plant world after cellulose. Both starch and cellulose are classified as glucose-based polymers. However, these two differ in the orientation of the linkages between the glucose subunits (Jacques et al., 2003). Starch is not fermented directly by many microorganisms due to the lack of essential starch-degrading or amylolytic enzymes to release glucose from the polysaccharide chains. Similarly, cellulose needs to be broken down into monosaccharides by microorganisms that produce cellulolytic enzymes before fermentation can take place.

As observed in Figure 4.6, ethanol was only produced after the substrate (soluble starch) was treated with amylase. In contrast, in the absence of the enzyme, no ethanol was detected.

This shows that amylase is important in the alcoholic fermentation process and in this case the production of ethanol. The enzyme is needed for the conversion of starch. These results correlate with the graph of glucose concentration (Figure 4.7), where glucose was only detected in media that contained starch with enzyme. It was noted that the conversion of starch into glucose occurred efficiently on the first day of fermentation as the maximum production of ethanol was recorded at 24 h, along with the depletion of glucose in the fermentation media. This indicates that the complete enzymatic hydrolysis occurred in the first 24 h of fermentation. The complete enzymatic hydrolysis requires a combination of  $\alpha$ -amylase (EC.3.2.1.1) and glucoamylase (EC.3.2.1.3).  $\alpha$ -amylase hydrolyzes the internal  $\alpha$ -1,4- bonds of amylose and amylopectin at random, producing free glucose and maltose (Kennedy et al., 1987). Glucoamylase hydrolyzes the terminal 1-4- linked  $\alpha$ -D-glucopyranosyl units from the non-reducing end of starch chains, resulting in the release of  $\beta$ -D-glucose (Belshaw & Williamson, 1993; Sauer et al., 2000). Based on the results obtained in this study, *C. glabrata* was shown to be capable of utilizing starch in the presence of amylase, with an ethanol yield of 56.33% of the theoretical value. This observation suggests that *C. glabrata* does not produce amylolytic enzymes to breakdown starch in the fermentation broth.

A similar profile was observed in the broth containing cellulose, where ethanol was only detected in the broth supplemented with cellulase. A maximum ethanol production of 8.83 g/l and a yield of 56.33% of the theoretical value were recorded. This outcome indicates that cellulase is crucial for the conversion of cellulose to ethanol, as supported by the findings of Fox et al. (2012) where the cellulase action on insoluble cellulosic substrates is modeled as a heterogenous biocatalytic reaction. The reaction basically involves endo- and exocellulases

that either interact directly with soluble cello-oligosaccharides or form complexes with insoluble cellulose chains. Cello-oligosaccharides are saccharides that consist of 2 to 6 glucose by  $\beta$ -1-4-linkages, containing mainly cellobiose (Otsuka et al., 2004; Zhao et al., 2009). In addition to glucose, cellobiose is another sugar that was also monitored in this study. Cellobiose is an intermediate product from the breakdown of cellulose and can be further converted into simple sugar such as glucose during fermentation to produce ethanol, by  $\beta$ -glucosidase (Philippidis & Smith, 1995). In this study, the concentration of cellobiose was observed to gradually increase in the first 12 h, indicating active enzymatic hydrolysis at the early stage of fermentation. Moreover, the depletion of cellobiose indicates its conversion to glucose over time. Our results show that *C. glabrata* is unable to breakdown cellulose and requires extracellular cellulase to convert cellulose into fermentable sugar.

In this study, *C. glabrata* was also being evaluated for biomass production. Total sago effluent was chosen as a substrate in the SSF process. Total sago effluent is a waste generated from the sago mill industry and are considered as non-food raw materials and do not compete with food supplies (Thompson & Meyer, 2012). Thus, ethanol produced from sago waste is classified as a second-generation biofuel. The sago palm is one of the most developed crops in Sarawak and its wastes can be found abundantly. Proper treatment of these wastes could minimize the problem of industrial waste accumulation. One of the possible solutions is by making them the substrate for bioethanol production.

Prior to the SSF process, composition analysis was conducted to determine the percentage of lignocellulosic components in total sago effluent. The composition of lignocellulosic

material varies based on the sago species, variety, growth conditions and maturity (Kim et al., 2010; Tye et al., 2016). Production of ethanol from lignocellulosic materials depends on the type of biomass, where a higher ethanol yield results from a high content of cellulose and hemicellulose but a low lignin content (Tye et al., 2016). In the current study, total sago effluent was found to be composed of sago effluent hydrolysate and sago hampas, with 97.14% water component (hydrolysate). Approximately 2.23% of the hydrolysate is starch, which can be converted to fermentable sugar through enzymatic hydrolysis. The rest are categorized as solid components or sago hampas. Further composition analysis was performed on sago hampas to quantify the lignocellulosic materials. Table 4.2 shows that dried sago hampas used in this study contains 52.03% starch, 27.82% cellulose, 5.32% hemicellulose and 3% lignin. The results obtained are comparable to previous findings by Hung et al. (2018), where the sago hampas was reported to contain 55.4% starch, 23.6% cellulose, 9.1% hemicellulose and 4.0% lignin. On top of that, the starch content recorded in this study was comparable with other reports, in the range of 30% - 50% (Awg-Adeni et al., 2010). The amount of cellulose and hemicellulose in biomass are the key in biofuel production, as they are convertible into bioethanol (Malherbe & Cloete, 2003). Therefore, total sago effluent is considered a potential substrate for bioethanol production due to its high content of lignocellulosic materials.

Biochemical conversion is a crucial process in the production of bioethanol, mainly due to the high selectivity and efficiency of biomass conversion (Sims et al., 2010; Achinas & Euverink, 2016). For instance, enzymes which act as biocatalyst is being applied during the hydrolysis of polysaccharides (Tye et al., 2016). These enzymes are important to release monosaccharides from polysaccharides located in the cell wall of plants. Untreated biomass

is difficult to be broken down by enzymes, requiring higher doses of hydrolytic enzymes such as amylases and cellulases (Sindhu et al., 2016). D-glucose is the main product derived from the hydrolysis of lignocellulose, mainly from cellulose. Meanwhile, hexose sugars (D-glucose, D-mannose, L-rhamnose) and pentose sugars (D-xylose, L-arabinose) are obtained from hemicellulose (El-Naggar et al., 2014). These sugars are subsequently converted into ethanol during fermentation. However, the conversion relies upon the type of raw material used and the pretreatment methods applied (Choudhary et al., 2017).

Enzymatic hydrolysis and fermentation can be carried out separately as Separate Hydrolysis and Fermentation (SHF) or at the same time in Simultaneous Saccharification and Fermentation (SSF). In this study, SSF was performed by combining the saccharification and fermentation process in a single reactor with the same fermentation media. SSF is reported to be the most promising process fermentation methods to produce ethanol from lignocellulosic biomass. Its advantages include higher reaction rates, higher yields and greater ethanol concentrations if compared to SHF (Tomás-Pejó et al., 2008). These lead to reductions in cost and inhibitors, at the same time an increase in hydrolysis rate (Foust et al., 2009). Based on Figure 4.15, a steep decrease of total sugar content during the first 6 h indicates the substrates present in the fermentation broth were rapidly fermented into ethanol by *C. glabrata*. Despite that, a small amount of sugar remained in the broth as the total sugar content stayed constant until the end of the SSF. The same observations were also reported by Hung et al. (2018), as the residual carbohydrates in the fermentation broth indicates that not all sugars were converted to ethanol. In addition, the results obtained in the current study show the presence of other hexose sugars in the fermentation broth besides glucose. Total carbohydrate content is commonly determined via PSA or Phenol-Sulfuric acid assay. PSA

has been reported as the most reliable quantitative assay for carbohydrate estimation (Dubois et al., 1956; Masuko et al., 2005). According to Nielsen (2010), the basic principle of this method is that carbohydrates when dehydrated by reaction of concentrated sulfuric acid, produce furfural derivatives, which further reacts with phenol to form a yellow brown-coloured product, that can be measured spectrophotometrically (Appendix, Figure 3). The darker of the colour formed, the higher of the absorbance reading, which shows that the sample has higher carbohydrate content. In this study, the colour of sample was observed to be darker at 0 h compared to other sampling hours. This reflects the total carbohydrate content where the highest carbohydrate content was found at the beginning of SSF.

Further HPLC analysis was performed to determine the presence of ethanol and carbohydrates from total sago effluent (Vincent et al., 2014). As shown in Figure 4.16, ethanol was produced at the beginning of SSF process, with a concentration of 2.22 g/l at 6 h, indicating that the glucose released from total sago effluent was converted into ethanol simultaneously. The main purpose of conducting SSF was successfully achieved as the time taken for the whole process was shortened (Foust et al., 2009). The increase in ethanol production is highly correlated with the steep decrease in glucose concentration, where the glucose was consumed by *C. glabrata* to produce ethanol during the first 6 h. The glucose produced from hydrolysis was rapidly fermented into ethanol up to 12 h and this has resulted in the depletion of glucose until the end of the SSF. This, in turn, could reduce the problems caused by product inhibition in the SSF process (Chen et al., 2007). After the depletion of glucose at 6 h, ethanol concentration continued to increase until its maximum production at 24 h. The process of saccharification and fermentation could have carried on in low concentrations. It is possible that the glucose generated was immediately converted into

ethanol, making it undetected by HPLC. After 24 h, ethanol production slowly decreased, indicating depletion in sugars to be converted into ethanol. In other words, the maximum ethanol production coincided with glucose depletion in the fermentation broth. Our results showed that 5.54 g/l of bioethanol was produced by *C. glabrata* in a working volume of 150 ml total sago effluent that contain sago hampas and sago effluent. Several studies on the production of bioethanol from sago, especially sago hampas, have been carried out. Hung et al. (2018) reported on the potential of sago hampas as feedstock for bioethanol production, with 79.65% of theoretical ethanol yield and *S. cerevisiae* as a fermenting agent.

The highest glucose concentration was recorded at the beginning of SSF, indicating the most efficient enzymatic hydrolysis of starchy materials and cellulosic components from sago hampas and sago hydrolysate. Glucose, the main sugar that is fermented by *C. glabrata* can be derived from enzymatic hydrolysis of starchy and lignocellulosic components. Based on composition analysis, total sago effluent is made up of mostly starch components, which is present in both sago hampas and sago hydrolysate. The glucose that was detected in the fermentation media would have been derived from starch materials, and not lignocellulosic materials.

The complete breakdown of cellulose into glucose involves extracellular cellulase. Cellobiose is a disaccharide that is an intermediate product of the hydrolysis of cellulose. Based on the results, the highest concentration of cellobiose was found at the beginning of SSF, indicating the enzymatic hydrolysis of cellulose into cellobiose. As reported by Yang et al. (2011), enzymatic hydrolysis of cellulose deconstructs cellulose and other carbohydrate

polymers into fermentable sugars such as glucose and/or oligomers, which can be further converted into biofuel. Cellulase consists of three main components, namely 1, 4- $\beta$ -D-glucanase, 1, 4- $\beta$ -D-xylanase and  $\beta$ -D-glucosidase. Endoglucanases randomly attack cellulose chains to produce polysaccharides of a short length. Exoglucanases then attach to the non-reducing ends of these short chains, removing the cellobiose moieties. Subsequently,  $\beta$ -D-glucosidases cleave cellobiose into 2 glucose units (Lynd et al., 2002). The depletion of cellobiose denotes effective enzyme activity in the fermentation broth. However, cellobiose was only detected in low concentrations, and this in turn could have resulted in a low yield of glucose.

During SSF, the presence of the sugars such as xylose and arabinose were recorded and this is an indicator of effective enzymatic hydrolysis of typical lignocellulosic materials (Im et al., 2016). Xylose and arabinose are the most abundant pentose sugars in hemicellulose (Oloffson et al., 2008; Bettiga et al., 2009). These sugars are released from xyloglucan, xylan, arabinan and arabinogalactan (substructures of pectin) from polysaccharides in plant cell (Battaglia et al., 2011). As shown in Figure 4.17, xylose was only detected at 12 h and the peak was found at 24 h. On the contrary, arabinose was detected from the beginning until the end of SSF. The concentration of arabinose in the initial stage of SSF was low. The degradation of pectin during the autoclaving process of media preparation could have resulted in the accumulation of arabinose at this stage. The concentration of arabinose concentration was found to increase during the first 12 h, indicating active breakdown of hemicellulose by xylanase into xylose and arabinose. After 24 h, the concentration of xylose and arabinose remained constant until the end of the SSF, evidence of the inability of *C. glabrata* to utilize both xylose and arabinose. Barnett et al. (2000) reported that *C. glabrata*

is only able to ferment glucose and trehalose. Our data showed a similar trend. Other studies have shown that only a few microorganisms are capable of fermenting five-carbon sugars (Wyman, 1999; Lynd et al., 2002; Wyman et al., 2005).

In this study, the presence of weak acids was also being monitored. Lactic acid and acetic acid are the by-products from the conversion of biomass into ethanol. The production of these acids in high concentration is an indicator of contamination, which could be caused by lactobacilli (Narendranath et al., 1997; Narendranath, 2003). Thus, the concentration of lactic acid must be closely monitored to avoid contamination and the resulting reduction in ethanol production. On top of that, it is important to control the accumulation of these acids to maintain the pH of the fermentation broth (Lin et al., 2012). The data obtained in this study shows low concentrations of lactic acid in the broth, indicating the SSF was contamination-free. Acetic acid is commonly found in hydrolysates and it basically comes from acetyl side-chains in hemicellulose (Dien et al., 2003). According to Vincent et al. (2015a), acetic acid is an important indicator in determining the efficiency of biomass hydrolysis. The authors reported an increase in acetic acid concentration in the fermentation broth that denotes the continuous degradation of biomass. As shown in Figure 4.18, acetic acid was detected in the range of 0.2 to 0.5 g/l, signifying biomass degradation throughout the SSF.

As shown in Table 4.3 and Figure 4.19, ethanol production by *C. glabrata* is the highest in glucose among all substrates tested, with 79.84% of theoretical yield. This makes *C. glabrata* a highly suitable candidate for glucose fermentation. On the contrary, the ability of *C.*

*glabrata* to ferment sucrose appears to be very poor, with a low ethanol production of approximately 1.64 g/l or 6.44% of theoretical yield. SPSS analysis was conducted to compare the ethanol yield of each substrate tested. Figure 4.19 reveals a significant difference in the theoretical ethanol yield between total sago effluent and other substrates except sucrose. In summary, *C. glabrata* produced 5.54 g/l of bioethanol (21.25% of theoretical value) from SSF of total sago effluent, in a working volume of 150 ml. *C. glabrata* could be used in bioethanol production because it has the ability to grow on lignocellulosic substrate such as total sago effluent (Robak & Balcerek, 2018). However, for industrial bioethanol production based on lignocellulose as raw material, the microorganisms must meet some requirements. For example, they must be capable of fermenting xylose and arabinose, not only glucose. Efficient conversion of xylose and arabinose into ethanol is important because these two sugars are the main products from hydrolysis of lignocellulose substrate. In this study, although *C. glabrata* showed a high fermentative activity for glucose, this strain is incapable of fermenting xylose and arabinose, resulting in low ethanol yield and thus making it less preferable in industrial bioethanol processes. The desired ethanol-fermenting microorganism also need to possess high ethanol yield (above 90% of theoretical yield) when using lignocellulosic biomass in bioethanol production (Zaldivar et al., 2001). Since *C. glabrata* yielded low ethanol content during SSF of total sago effluent, therefore it can be concluded that *C. glabrata* is inefficient and unsuitable for industrial bioethanol processes.

## CHAPTER 6

### GENERAL CONCLUSIONS AND RECOMMENDATIONS

#### 6.1 General Conclusions

Civilization and expanding industrial activities lead to a greater demand for energy. The rapid depletion of fossil fuel supplies such as coal and oil are undeniably would affect developing countries. This issue coupled with the climate change-related threats had encouraged scientist to search for alternative fuel sources. Biofuel such as bioethanol has the potential to become an important sustainable fuel in the future. Ethanol can be produced from various sources like sugar, starchy materials and lignocellulosic materials. First-generation bioethanol uses food feedstock such as starchy materials while second-generation bioethanol yield ethanol derived from biomass, called lignocellulosic materials. Nowadays, bioethanol from lignocellulosic material is gaining more recognition and researchers looking for potential sources of biomass substrates rather than focusing on starchy materials, which had to compete with food supplies. With their vast availability, lignocellulosic materials such as total sago effluent might be considered as a suitable alternative to the diminishing fossil fuels. In the meantime, researcher also looking for new strains that could be used as a fermentative organism with the purpose to increase the bioethanol yield.

The main purpose of this study was to evaluate the capability and efficiency of *C. glabrata* as a fermentative organism in producing ethanol. The other two objectives were focusing on the production of ethanol from simple sugars and polysaccharides. The results indicated that *C. glabrata* was capable of fermenting glucose efficiently (79.84% of theoretical yield) among simple sugars, which makes it a good candidate to be employed together with the

other common fermenting yeasts such as *Saccharomyces cerevisiae*. On the contrary, our results showed that *C. glabrata* does not possess the ability to break down the polysaccharides like starch and cellulose without the aid of enzymes such as amylases and cellulases. *C. glabrata* is also incapable of fermenting other important sugars such as xylose and arabinose.

SSF was conducted at the last stage of this study to evaluate the activities of *C. glabrata* in bioethanol fermentation from total sago effluent and the results indicated *C. glabrata* is only capable in fermenting glucose that have been broken down from starchy and lignocellulosic materials in fermentation media containing total sago effluent. *C. glabrata* produced 5.54 g/l of bioethanol (10.68% of theoretical value) from SSF of total sago effluent. Thus, it can be concluded that *C. glabrata* yielded low ethanol content during SSF of total sago effluent and therefore *C. glabrata* is not suitable candidate for industrial bioethanol processes.

## **6.2 Future Recommendations**

Lignocellulosic materials such as sago hampas are known to have three main components which are cellulose, hemicellulose and lignin. All three components have covalent cross-linkages between the polysaccharide and lignin, which can be separated by pretreatment. The main purpose of pretreatment is to break down the compact structure of the lignocellulosic materials and the lignin component will be disrupted, and cellulose fiber is exposed, which allow them to be more accessible to microbial or enzymatic reaction. Previous studies have reported different methods of pretreatment, including biological, chemical, mechanical, and thermal process. As previously noted, an ideal pretreatment

should be effective for various lignocellulosic substrates and generate less inhibitor. The cost also must be inexpensive, and the pretreatment should be able to remove lignin as much as possible and give minimal glucan loss.

Based on the literature review, fermentative organisms are important in bioethanol production to obtain high ethanol yield. This could be achieved by increasing the range of sugars consumed by *C. glabrata*. For example, genetic modification can be performed. The genetically engineered strain of *C. glabrata* must possess the ability to ferment pentose sugars such as xylose and arabinose, as these sugars cannot be consumed by *C. glabrata*. Therefore, ethanol production may be potentially enhanced as the fermentation of mixtures of hexoses and pentoses is carried out together.

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## APPENDICES

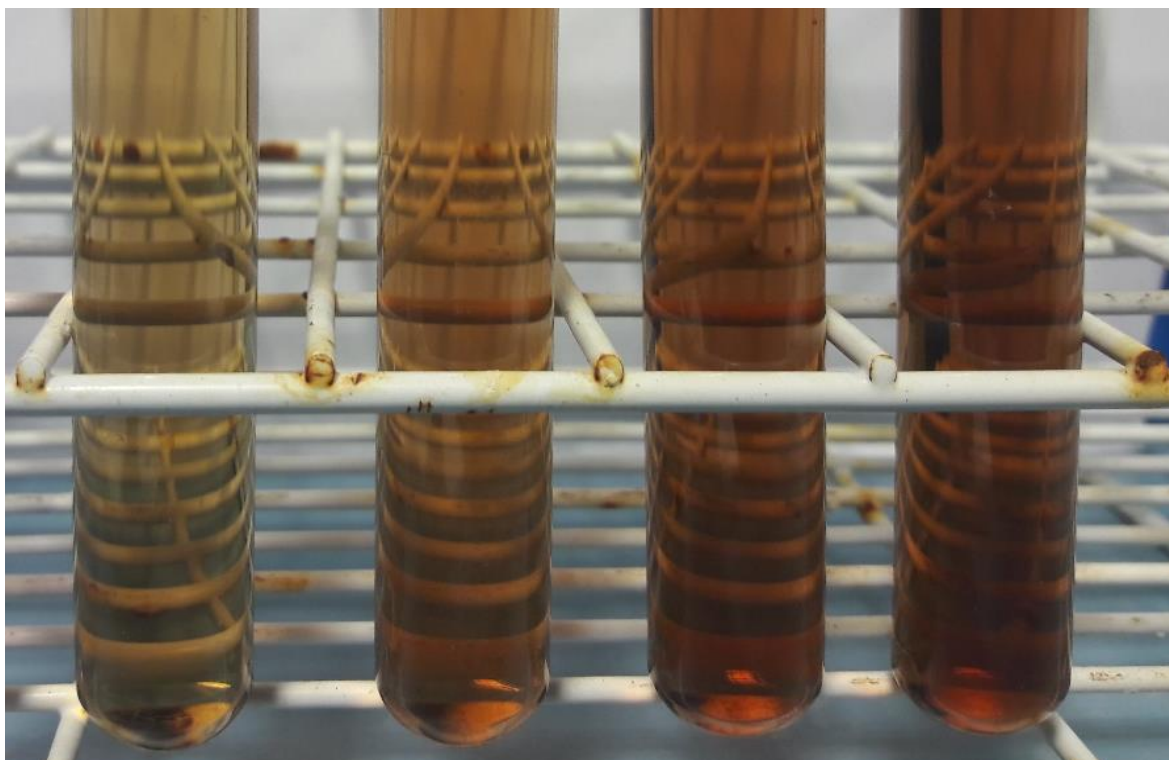


**Figure 1:** Colour development for standard curve of phenol sulphuric acid assay.

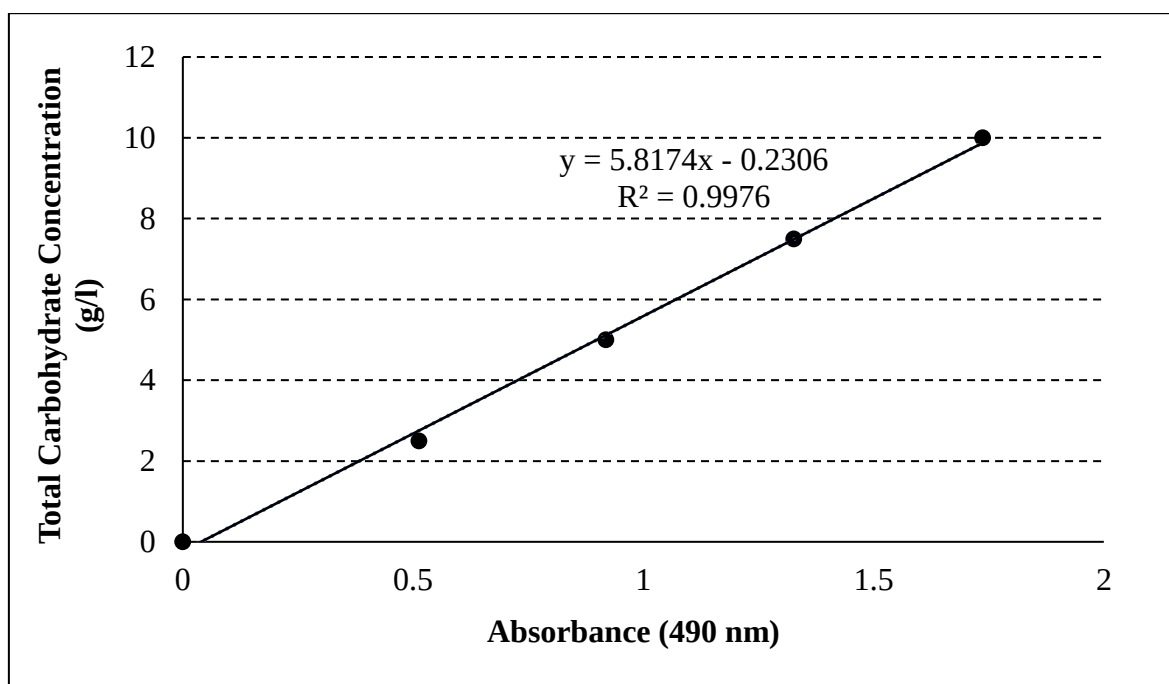


**Figure 2:** Standard curve of glucose for PSA.



**Figure 3:** Colour development of TSE sample for phenol sulphuric acid assay.

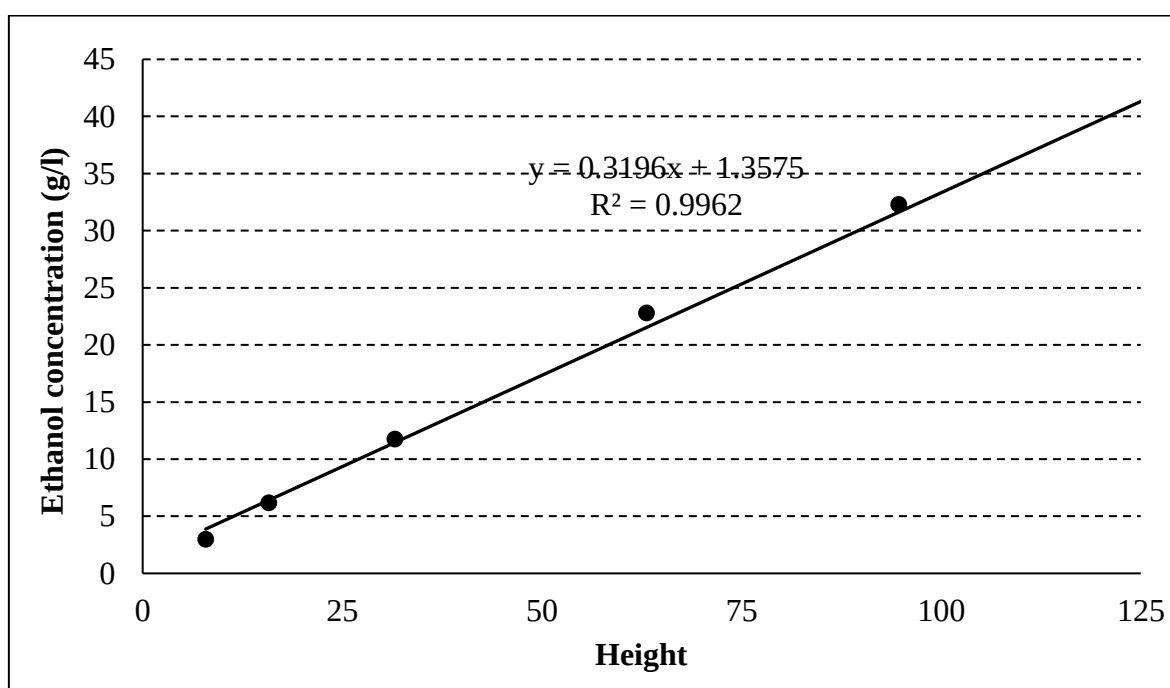


**Figure 4:** Standard curve for ethanol determination via HPLC.

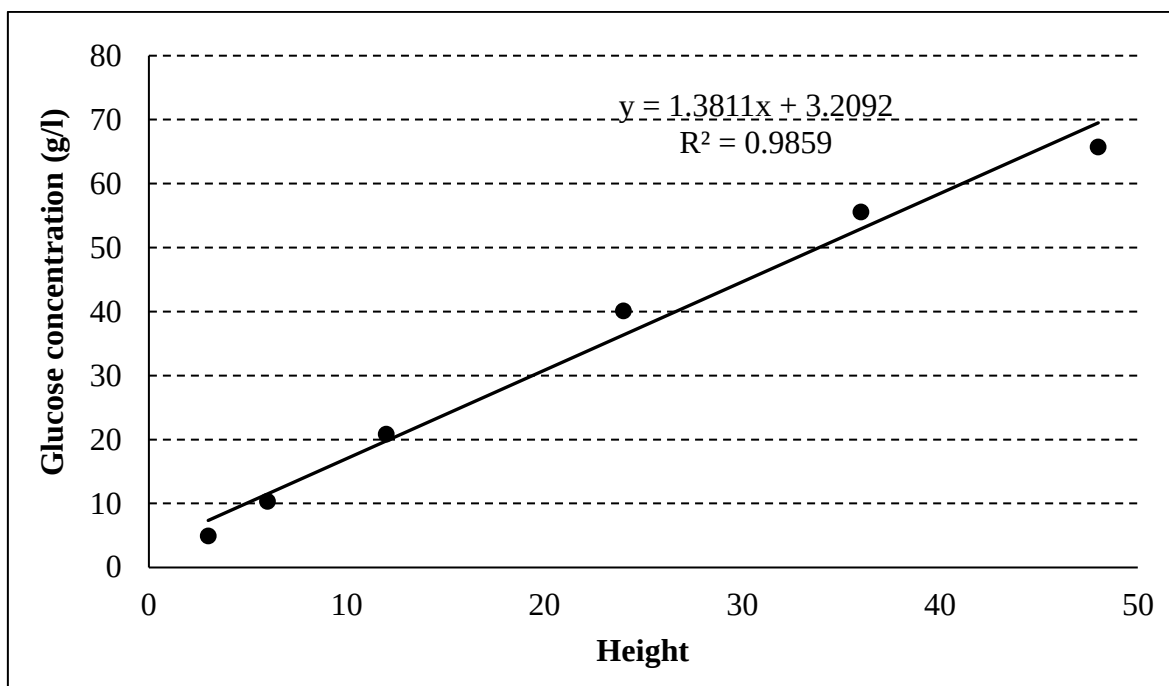


**Figure 5:** Standard curve for glucose/sucrose determination via HPLC.

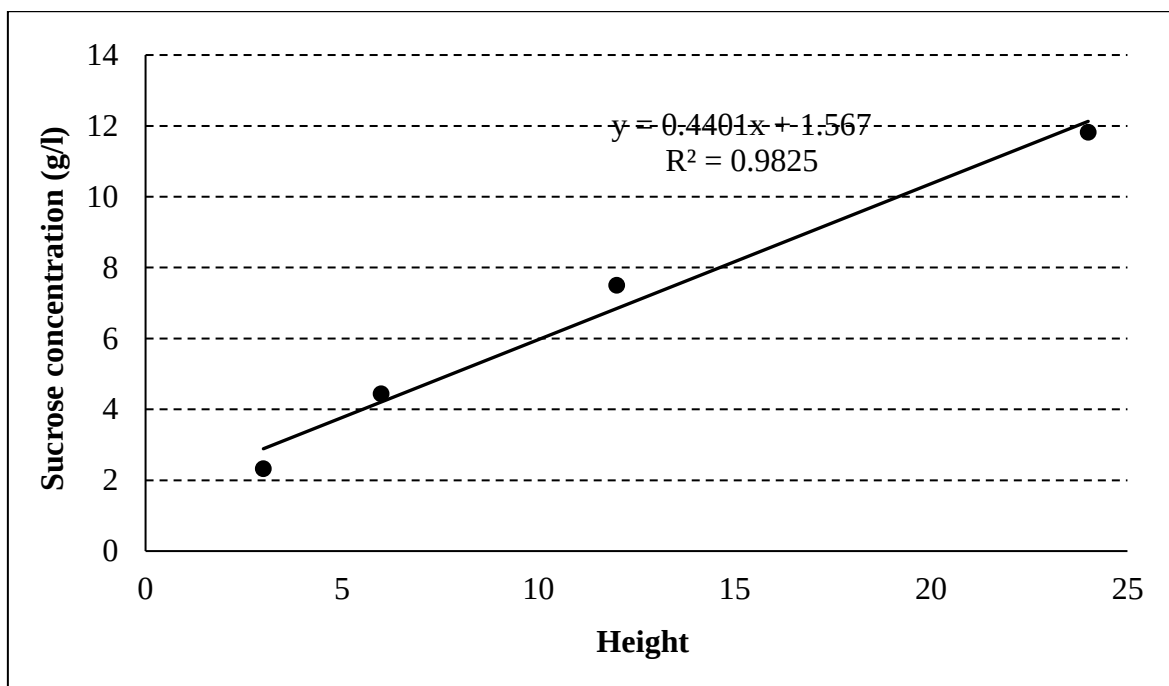


**Figure 6:** Standard curve for sucrose determination via HPLC.

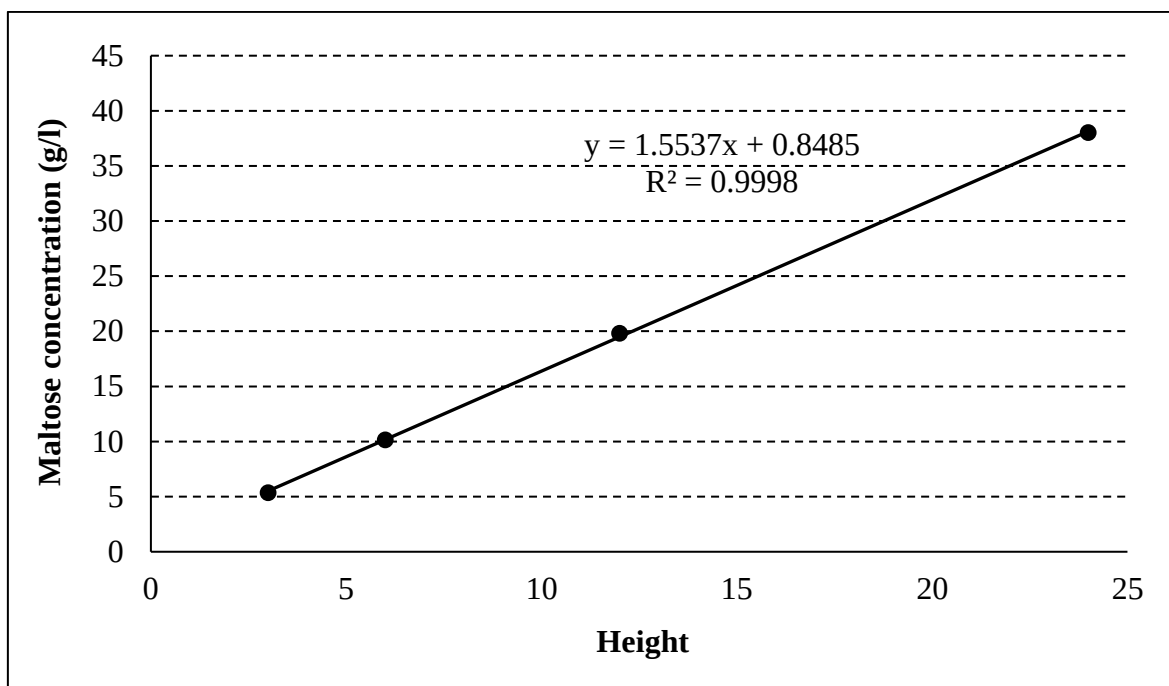


**Figure 7:** Standard curve for maltose determination via HPLC.

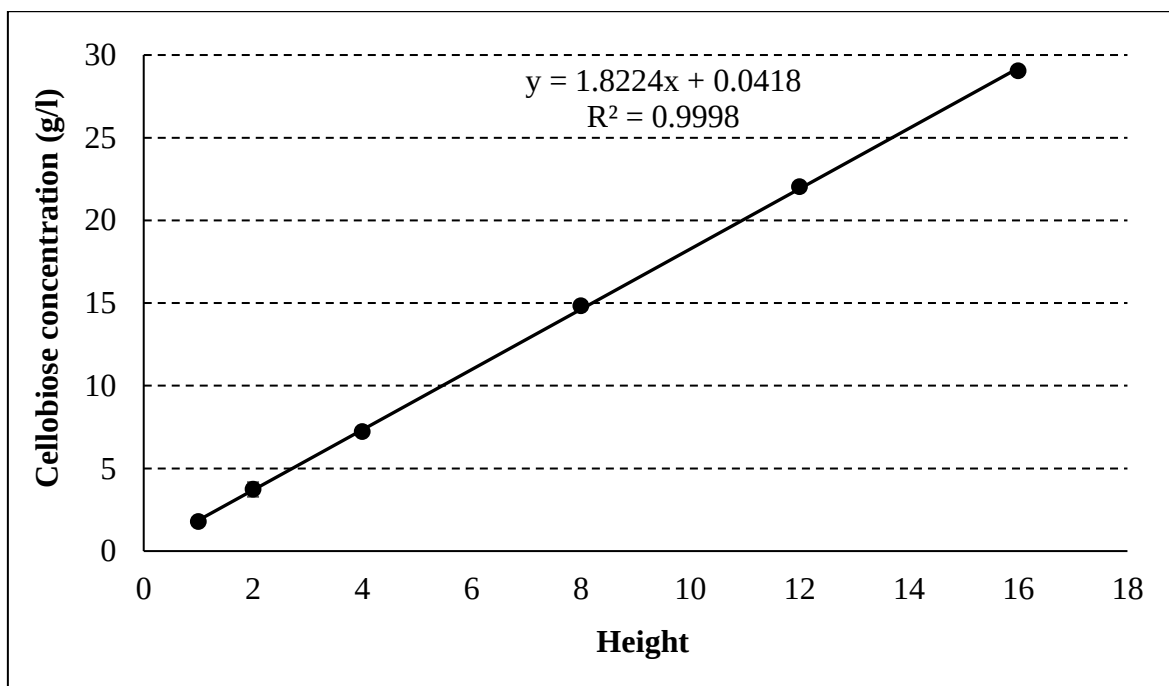


**Figure 8:** Standard curve for cellobiose determination via HPLC.

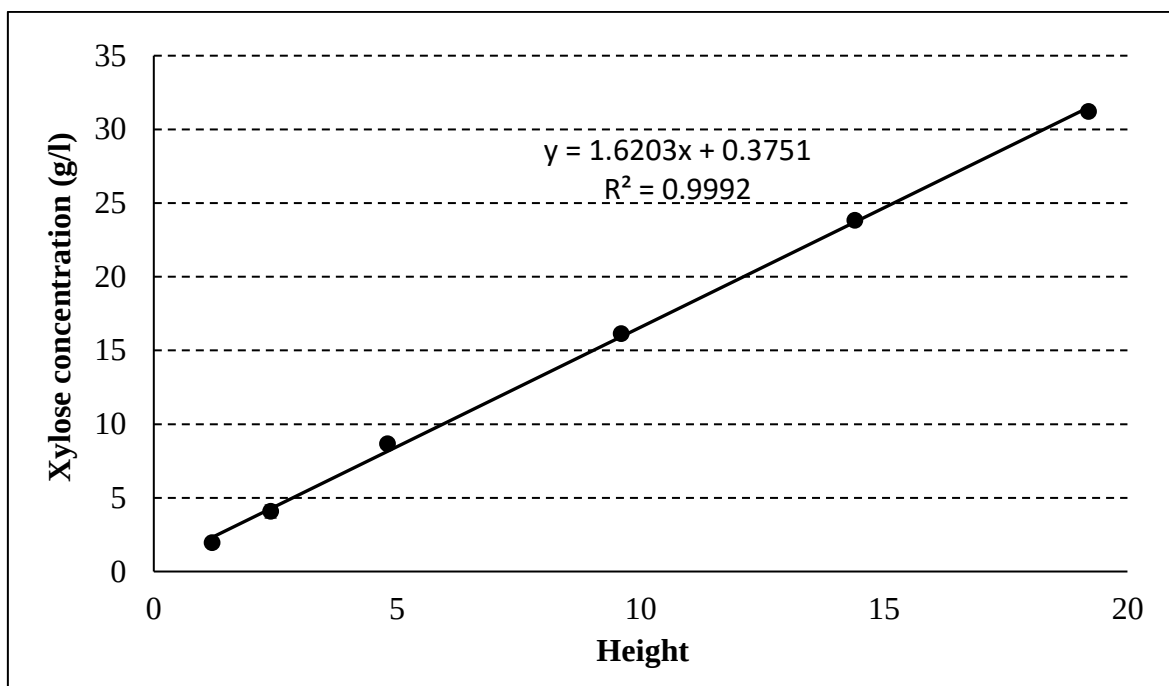


**Figure 9:** Standard curve for xylose determination via HPLC.

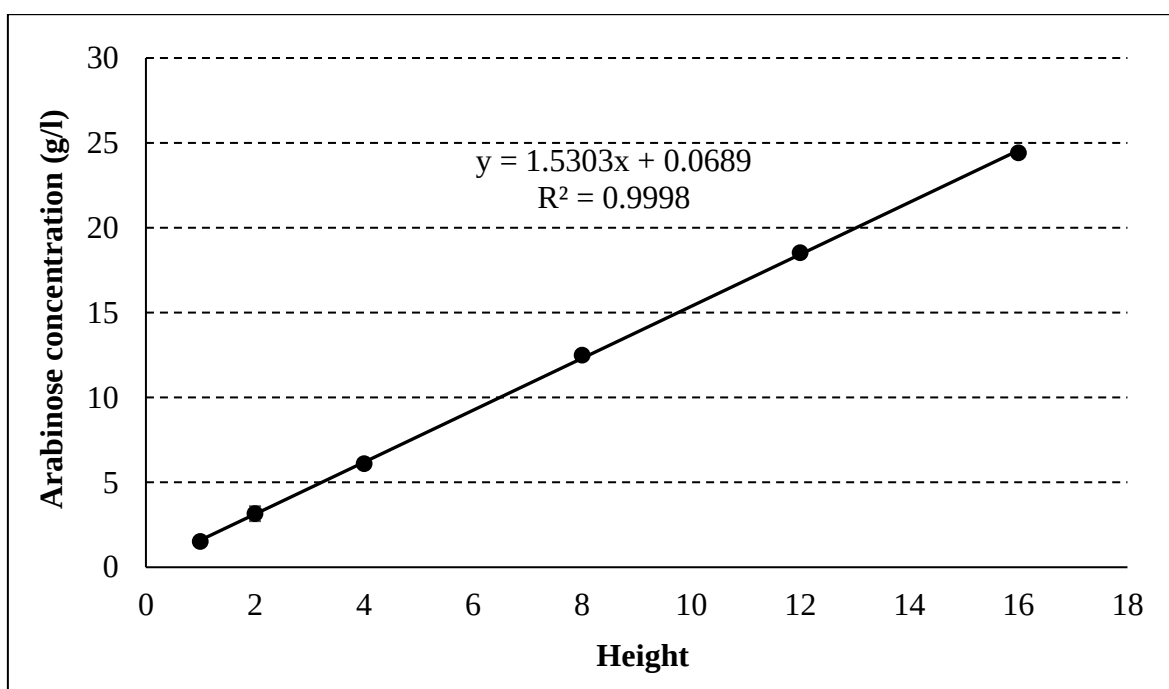


**Figure 10:** Standard curve for arabinose via HPLC.

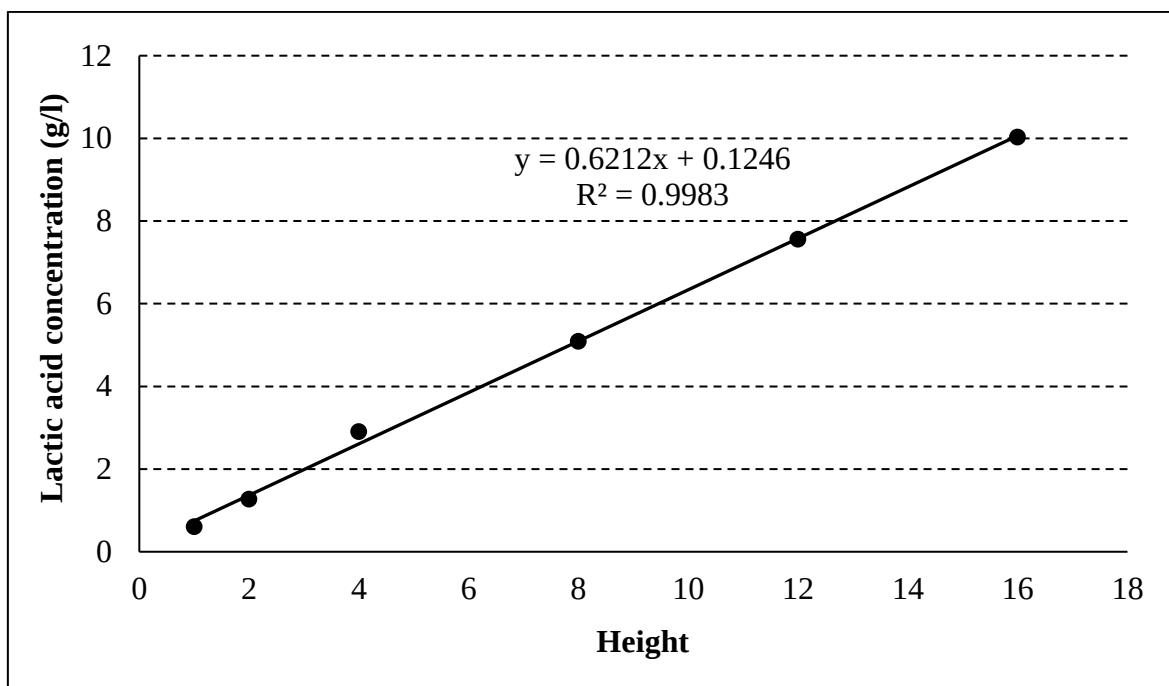


**Figure 11:** Standard curve for lactic acid via HPLC.

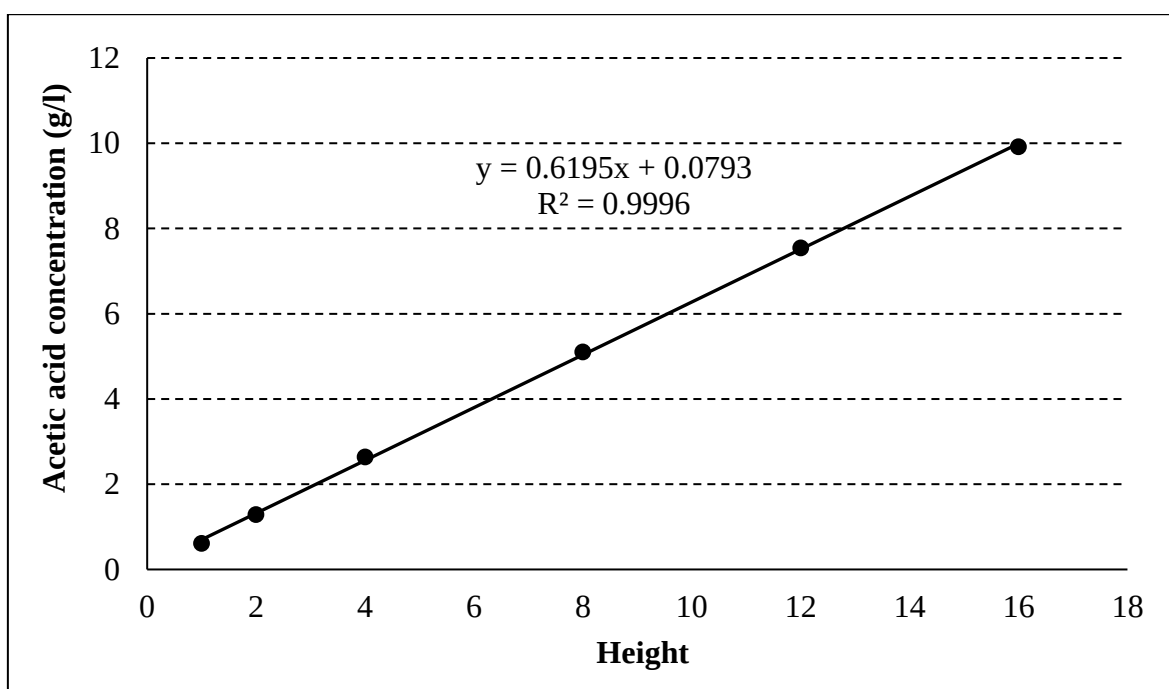


**Figure 12:** Standard curve for acetic acid via HPLC.

**Table 1:** Results of cell count of *Candida glabrata* for simple sugars and total sago effluent.

| Hour(s) | Glucose                | Maltose                | Sucrose                | Total sago effluent    |
|---------|------------------------|------------------------|------------------------|------------------------|
| 0       | 2.86E+08 ±<br>2.61E+07 | 2.00E+08 ±<br>3.21E+06 | 1.98E+08 ±<br>3.06E+06 | 2.59E+08 ±<br>7.23E+06 |
| 6       | 6.32E+08 ±<br>7.21E+06 | 2.44E+08 ±<br>1.22E+07 | 2.91E+08 ±<br>1.30E+07 | 6.80E+08 ±<br>1.52E+07 |
| 12      | 7.82E+08 ±<br>2.31E+07 | 2.47E+08 ±<br>1.10E+07 | 3.25E+08 ±<br>1.18E+07 | 6.94E+08 ±<br>6.35E+06 |
| 24      | 8.12E+08 ±<br>4.45E+07 | 2.55E+08 ±<br>1.36E+07 | 3.37E+08 ±<br>3.61E+06 | 6.86E+08 ±<br>2.08E+07 |
| 36      | 8.58E+08 ±<br>5.20E+07 | 2.61E+08 ±<br>3.61E+06 | 4.09E+08 ±<br>4.93E+06 | 6.93E+08 ±<br>2.20E+07 |
| 48      | 9.94E+08 ±<br>3.00E+06 | 2.94E+08 ±<br>6.03E+06 | 4.22E+08 ±<br>8.89E+06 | 6.89E+08 ±<br>6.03E+06 |
| 72      | 9.16E+08 ±<br>1.36E+07 | 3.32E+08 ±<br>1.24E+07 | 3.94E+08 ±<br>2.05E+07 | 7.26E+08 ±<br>1.38E+07 |
| 96      | 8.82E+08 ±<br>4.31E+07 | 3.35E+08 ±<br>8.08E+06 | 3.75E+08 ±<br>1.39E+07 | 7.12E+08 ±<br>1.57E+07 |
| 120     | 8.66E+08 ±<br>5.66E+07 | 3.33E+08 ±<br>1.44E+07 | 3.80E+08 ±<br>1.22E+07 | 6.27E+08 ±<br>1.15E+07 |

**Table 2:** Results of cell count of *C. glabrata* for complex sugars.

| Hour(s) | Starch with Enzyme     | Starch without Enzyme  | Cellulose with Enzyme  | Cellulose without money |
|---------|------------------------|------------------------|------------------------|-------------------------|
| 0       | 1.67E+08 ±<br>3.00E+07 | 1.99E+08 ±<br>1.47E+07 | 1.99E+08 ±<br>8.39E+06 | 1.29E+08 ±<br>1.21E+07  |
| 6       | 3.81E+08 ±<br>1.35E+07 | 2.32E+08 ±<br>8.50E+06 | 2.58E+08 ±<br>5.03E+06 | 1.89E+08 ±<br>2.16E+07  |
| 12      | 6.58E+08 ±<br>1.31E+07 | 2.58E+08 ±<br>1.99E+07 | 3.74E+08 ±<br>1.46E+07 | 2.41E+08 ±<br>1.90E+07  |
| 24      | 8.38E+08 ±<br>4.57E+07 | 2.47E+08 ±<br>6.24E+06 | 4.19E+08 ±<br>4.63E+07 | 2.67E+08 ±<br>1.26E+07  |
| 36      | 8.94E+08 ±<br>3.87E+07 | 2.67E+08 ±<br>8.72E+06 | 4.54E+08 ±<br>4.05E+07 | 2.67E+08 ±<br>8.74E+06  |
| 48      | 8.47E+08 ±<br>3.74E+07 | 2.86E+08 ±<br>8.54E+06 | 4.10E+08 ±<br>1.57E+07 | 2.79E+08 ±<br>7.23E+06  |
| 72      | 8.17E+08 ±<br>2.75E+07 | 3.02E+08 ±<br>1.93E+07 | 3.66E+08 ±<br>1.84E+07 | 3.20E+08 ±<br>1.71E+07  |
| 96      | 7.63E+08 ±<br>2.08E+07 | 2.79E+08 ±<br>3.06E+06 | 3.25E+08 ±<br>1.05E+07 | 3.18E+08 ±<br>1.12E+07  |
| 120     | 7.07E+08 ±<br>1.26E+07 | 2.79E+08 ±<br>2.08E+06 | 2.98E+08 ±<br>1.53E+06 | 2.97E+08 ±<br>7.02E+06  |

**Table 3:** Results of fermentation for glucose.

| Hour(s) | Ethanol (g/l) | Glucose (g/l) | Lactic acid (g/l) | Acetic acid (g/l) |
|---------|---------------|---------------|-------------------|-------------------|
| 0       | 0.23 ± 0.13   | 46.44 ± 2.33  | 0.36 ± 0.15       | 0.00 ± 0.00       |
| 6       | 6.67 ± 0.24   | 35.12 ± 0.84  | 1.94 ± 0.06       | 0.00 ± 0.00       |
| 12      | 11.29 ± 0.96  | 25.67 ± 0.79  | 3.52 ± 0.44       | 0.00 ± 0.00       |
| 24      | 16.12 ± 0.61  | 15.64 ± 0.63  | 3.71 ± 0.32       | 0.00 ± 0.00       |
| 36      | 18.78 ± 0.16  | 8.31 ± 0.50   | 3.63 ± 0.28       | 0.34 ± 0.03       |
| 48      | 19.69 ± 0.39  | 0.00 ± 0.00   | 3.60 ± 0.16       | 0.38 ± 0.01       |
| 72      | 20.40 ± 0.30  | 0.00 ± 0.00   | 3.45 ± 0.50       | 0.40 ± 0.11       |
| 96      | 19.91 ± 0.92  | 0.00 ± 0.00   | 3.17 ± 0.64       | 0.41 ± 0.08       |
| 120     | 19.35 ± 0.52  | 0.00 ± 0.00   | 3.20 ± 0.66       | 0.36 ± 0.19       |

**Table 4:** Results of fermentation for maltose.

| Hour(s) | Ethanol (g/l) | Maltose (g/l) | Lactic acid (g/l) | Acetic acid (g/l) |
|---------|---------------|---------------|-------------------|-------------------|
| 0       | 0.00 ± 0.00   | 49.21 ± 0.16  | 0.00 ± 0.00       | 0.14 ± 0.01       |
| 6       | 0.00 ± 0.00   | 49.62 ± 0.16  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 12      | 0.00 ± 0.00   | 48.92 ± 0.55  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 24      | 0.00 ± 0.00   | 49.36 ± 0.09  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 36      | 0.00 ± 0.00   | 49.34 ± 0.72  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 48      | 0.00 ± 0.00   | 49.01 ± 0.29  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 72      | 0.00 ± 0.00   | 49.58 ± 0.30  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 96      | 0.00 ± 0.00   | 49.15 ± 0.35  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 120     | 0.00 ± 0.00   | 49.20 ± 0.55  | 0.00 ± 0.00       | 0.00 ± 0.00       |

**Table 5:** Results of fermentation for sucrose.

| Hour(s) | Ethanol (g/l) | Sucrose (g/l) | Lactic acid (g/l) | Acetic acid (g/l) |
|---------|---------------|---------------|-------------------|-------------------|
| 0       | 0.00 ± 0.00   | 49.87 ± 2.22  | 0.00 ± 0.00       | 0.14 ± 0.01       |
| 6       | 1.64 ± 0.08   | 48.63 ± 1.50  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 12      | 1.54 ± 0.15   | 48.61 ± 0.87  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 24      | 1.35 ± 0.14   | 49.32 ± 1.20  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 36      | 1.06 ± 0.21   | 48.82 ± 0.77  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 48      | 0.84 ± 0.51   | 48.39 ± 1.09  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 72      | 0.39 ± 0.67   | 48.75 ± 0.85  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 96      | 0.35 ± 0.61   | 48.53 ± 1.42  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 120     | 0.32 ± 0.56   | 48.46 ± 1.67  | 0.00 ± 0.00       | 0.00 ± 0.00       |

**Table 6:** Results of fermentation for starch with enzyme.

| Hour(s) | Ethanol (g/l) | Glucose (g/l) | Lactic acid (g/l) | Acetic acid (g/l) |
|---------|---------------|---------------|-------------------|-------------------|
| 0       | 0.00 ± 0.00   | 53.69 ± 5.37  | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 6       | 1.62 ± 0.79   | 11.71 ± 3.84  | 0.09 ± 0.01       | 0.00 ± 0.00       |
| 12      | 9.43 ± 1.37   | 0.00 ± 0.00   | 0.18 ± 0.01       | 0.00 ± 0.00       |
| 24      | 15.48 ± 2.11  | 0.00 ± 0.00   | 0.10 ± 0.03       | 0.00 ± 0.00       |
| 36      | 13.68 ± 0.93  | 0.00 ± 0.00   | 0.15 ± 0.02       | 0.00 ± 0.00       |
| 48      | 13.39 ± 1.16  | 0.00 ± 0.00   | 0.12 ± 0.03       | 0.00 ± 0.00       |
| 72      | 13.10 ± 0.72  | 0.00 ± 0.00   | 0.05 ± 0.08       | 0.00 ± 0.00       |
| 96      | 13.32 ± 0.62  | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.10 ± 0.09       |
| 120     | 11.53 ± 1.89  | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.15 ± 0.00       |

**Table 7:** Results of fermentation for starch without enzyme.

| Hour(s) | Ethanol (g/l) | Glucose (g/l) | Lactic acid (g/l) | Acetic acid (g/l) |
|---------|---------------|---------------|-------------------|-------------------|
| 0       | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 6       | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 12      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 24      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 36      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 48      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 72      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 96      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 120     | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00       | 0.00 ± 0.00       |

**Table 8:** Results of fermentation for cellulose with enzyme.

| Hour(s) | Ethanol (g/l) | Glucose (g/l) | Cellobiose (g/l) | Lactic acid (g/l) | Acetic acid (g/l) |
|---------|---------------|---------------|------------------|-------------------|-------------------|
| 0       | 0.43 ± 0.02   | 1.06 ± 0.09   | 3.73 ± 0.07      | 0.20 ± 0.04       | 0.27 ± 0.05       |
| 6       | 1.55 ± 0.26   | 0.83 ± 0.26   | 5.96 ± 0.14      | 0.15 ± 0.02       | 0.00 ± 0.00       |
| 12      | 2.64 ± 0.17   | 0.62 ± 0.09   | 6.30 ± 0.05      | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 24      | 4.93 ± 0.80   | 0.00 ± 0.00   | 5.40 ± 0.23      | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 36      | 8.83 ± 0.76   | 0.00 ± 0.00   | 0.00 ± 0.00      | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 48      | 8.32 ± 0.19   | 0.00 ± 0.00   | 0.00 ± 0.00      | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 72      | 7.54 ± 0.86   | 0.00 ± 0.00   | 0.00 ± 0.00      | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 96      | 7.53 ± 0.32   | 0.00 ± 0.00   | 0.00 ± 0.00      | 0.00 ± 0.00       | 0.00 ± 0.00       |
| 120     | 7.53 ± 0.15   | 0.00 ± 0.00   | 0.00 ± 0.00      | 0.00 ± 0.00       | 0.00 ± 0.00       |

**Table 9:** Results of fermentation for cellulose without enzyme.

| Hour(s) | Ethanol (g/l) | Glucose (g/l) | Lactic acid<br>(g/l) | Acetic acid<br>(g/l) |
|---------|---------------|---------------|----------------------|----------------------|
| 0       | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 6       | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 12      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 24      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 36      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 48      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 72      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 96      | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |
| 120     | 0.00 ± 0.00   | 0.00 ± 0.00   | 0.00 ± 0.00          | 0.00 ± 0.00          |

**Table 10:** Results of SSF for total sago effluent.

| Hour(s) | Total carbohydrate content (g/l) | Ethanol (g/l) | Glucose (g/l) | Cellobiose (g/l) | Xylose (g/l) | Arabinose (g/l) | Lactic acid (g/l) | Acetic acid (g/l) |
|---------|----------------------------------|---------------|---------------|------------------|--------------|-----------------|-------------------|-------------------|
| 0       | 20.57 ± 0.24                     | 0.00 ± 0.00   | 16.47 ± 0.32  | 4.32 ± 0.16      | 0.00 ± 0.00  | 0.40 ± 0.17     | 0.12 ± 0.01       | 0.46 ± 0.01       |
| 6       | 4.98 ± 0.44                      | 2.22 ± 0.18   | 0.56 ± 0.50   | 4.52 ± 0.01      | 0.00 ± 0.00  | 0.62 ± 0.04     | 0.10 ± 0.02       | 0.37 ± 0.01       |
| 12      | 4.02 ± 0.13                      | 4.21 ± 0.36   | 0.00 ± 0.00   | 2.39 ± 0.18      | 0.19 ± 0.05  | 0.80 ± 0.12     | 0.17 ± 0.07       | 0.37 ± 0.04       |
| 24      | 4.03 ± 0.26                      | 5.44 ± 0.09   | 0.00 ± 0.00   | 1.62 ± 0.03      | 0.28 ± 0.01  | 0.85 ± 0.03     | 0.12 ± 0.01       | 0.27 ± 0.07       |
| 36      | 4.03 ± 0.22                      | 5.18 ± 0.04   | 0.00 ± 0.00   | 1.61 ± 0.02      | 0.27 ± 0.01  | 0.87 ± 0.05     | 0.13 ± 0.03       | 0.24 ± 0.03       |
| 48      | 3.80 ± 0.13                      | 4.90 ± 0.17   | 0.00 ± 0.00   | 1.63 ± 0.00      | 0.27 ± 0.01  | 0.89 ± 0.04     | 0.14 ± 0.02       | 0.27 ± 0.04       |
| 72      | 3.79 ± 0.30                      | 4.70 ± 0.50   | 0.00 ± 0.00   | 1.67 ± 0.03      | 0.26 ± 0.01  | 0.91 ± 0.03     | 0.13 ± 0.02       | 0.33 ± 0.05       |

**Table 11:** One Way ANOVA tests between eight independent experiment.

|                           | Sum of Squares | df | Mean Square | F       | Sig. |
|---------------------------|----------------|----|-------------|---------|------|
| Between Groups (Combined) | 18549.357      | 7  | 2649.908    | 308.025 | .000 |
| Within Groups             | 137.646        | 16 | 8.603       |         |      |
| Total                     | 18687.003      | 23 |             |         |      |

**Table 12:** *Post hoc* tests between eight independent experiment.

| (I)<br>SUBSTRATE | (J)<br>SUBSTRATE               | Mean<br>Difference<br>(I-J) | Std.<br>Error | Sig.  | 95% Confidence Interval<br>Lower Bound Upper Bound |          |
|------------------|--------------------------------|-----------------------------|---------------|-------|----------------------------------------------------|----------|
| Glucose          | Maltose                        | 79.84380*                   | 2.39484       | .000  | 71.5525                                            | 88.1351  |
|                  | Sucrose                        | 73.40683*                   | 2.39484       | .000  | 65.1155                                            | 81.6981  |
|                  | Starch with<br>Enzyme          | 24.75984*                   | 2.39484       | .000  | 16.4685                                            | 33.0511  |
|                  | Starch without<br>Enzyme       | 79.84380*                   | 2.39484       | .000  | 71.5525                                            | 88.1351  |
|                  | Cellulose with<br>Enzyme       | 48.43545*                   | 2.39484       | .000  | 40.1442                                            | 56.7267  |
|                  | Cellulose<br>without<br>Enzyme | 79.84380*                   | 2.39484       | .000  | 71.5525                                            | 88.1351  |
|                  | Total sago<br>effluent         | 58.59065*                   | 2.39484       | .000  | 50.2994                                            | 66.8820  |
| Maltose          | Glucose                        | -79.84380*                  | 2.39484       | .000  | -88.1351                                           | -71.5525 |
|                  | Sucrose                        | -6.43697                    | 2.39484       | .196  | -14.7283                                           | 1.8543   |
|                  | Starch with<br>Enzyme          | -55.08396*                  | 2.39484       | .000  | -63.3753                                           | -46.7927 |
|                  | Starch without<br>Enzyme       | .00000                      | 2.39484       | 1.000 | -8.2913                                            | 8.2913   |
|                  | Cellulose with<br>Enzyme       | -31.40835*                  | 2.39484       | .000  | -39.6997                                           | -23.1171 |

**Table 12** continued

|                       |                          |            |         |       |          |          |
|-----------------------|--------------------------|------------|---------|-------|----------|----------|
|                       | Cellulose without Enzyme | .00000     | 2.39484 | 1.000 | -8.2913  | 8.2913   |
|                       | Total sago effluent      | -21.25315* | 2.39484 | .000  | -29.5445 | -12.9619 |
| Sucrose               | Glucose                  | -73.40683* | 2.39484 | .000  | -81.6981 | -65.1155 |
|                       | Maltose                  | 6.43697    | 2.39484 | .196  | -1.8543  | 14.7283  |
|                       | Starch with Enzyme       | -48.64699* | 2.39484 | .000  | -56.9383 | -40.3557 |
|                       | Starch without Enzyme    | 6.43697    | 2.39484 | .196  | -1.8543  | 14.7283  |
|                       | Cellulose with Enzyme    | -24.97138* | 2.39484 | .000  | -33.2627 | -16.6801 |
|                       | Cellulose without Enzyme | 6.43697    | 2.39484 | .196  | -1.8543  | 14.7283  |
|                       | Total sago effluent      | -14.81618* | 2.39484 | .000  | -23.1075 | -6.5249  |
| Starch with Enzyme    | Glucose                  | -24.75984* | 2.39484 | .000  | -33.0511 | -16.4685 |
|                       | Maltose                  | 55.08396*  | 2.39484 | .000  | 46.7927  | 63.3753  |
|                       | Sucrose                  | 48.64699*  | 2.39484 | .000  | 40.3557  | 56.9383  |
|                       | Starch without Enzyme    | 55.08396*  | 2.39484 | .000  | 46.7927  | 63.3753  |
|                       | Cellulose with Enzyme    | 23.67561*  | 2.39484 | .000  | 15.3843  | 31.9669  |
|                       | Cellulose without Enzyme | 55.08396*  | 2.39484 | .000  | 46.7927  | 63.3753  |
|                       | Total sago effluent      | 33.83081*  | 2.39484 | .000  | 25.5395  | 42.1221  |
| Starch without Enzyme | Glucose                  | -79.84380* | 2.39484 | .000  | -88.1351 | -71.5525 |
|                       | Maltose                  | .00000     | 2.39484 | 1.000 | -8.2913  | 8.2913   |
|                       | Sucrose                  | -6.43697   | 2.39484 | .196  | -14.7283 | 1.8543   |
|                       | Starch with Enzyme       | -55.08396* | 2.39484 | .000  | -63.3753 | -46.7927 |

**Table 12** continued

|                          |                          |            |         |       |          |          |
|--------------------------|--------------------------|------------|---------|-------|----------|----------|
|                          | Cellulose with Enzyme    | -31.40835* | 2.39484 | .000  | -39.6997 | -23.1171 |
|                          | Cellulose without Enzyme | .00000     | 2.39484 | 1.000 | -8.2913  | 8.2913   |
|                          | Total sago effluent      | -21.25315* | 2.39484 | .000  | -29.5445 | -12.9619 |
| Cellulose with Enzyme    | Glucose                  | -48.43545* | 2.39484 | .000  | -56.7267 | -40.1442 |
|                          | Maltose                  | 31.40835*  | 2.39484 | .000  | 23.1171  | 39.6997  |
|                          | Sucrose                  | 24.97138*  | 2.39484 | .000  | 16.6801  | 33.2627  |
|                          | Starch with Enzyme       | -23.67561* | 2.39484 | .000  | -31.9669 | -15.3843 |
|                          | Starch without Enzyme    | 31.40835*  | 2.39484 | .000  | 23.1171  | 39.6997  |
|                          | Cellulose without Enzyme | 31.40835*  | 2.39484 | .000  | 23.1171  | 39.6997  |
|                          | Total sago effluent      | 10.15520*  | 2.39484 | .011  | 1.8639   | 18.4465  |
| Cellulose without Enzyme | Glucose                  | -79.84380* | 2.39484 | .000  | -88.1351 | -71.5525 |
|                          | Maltose                  | .00000     | 2.39484 | 1.000 | -8.2913  | 8.2913   |
|                          | Sucrose                  | -6.43697   | 2.39484 | .196  | -14.7283 | 1.8543   |
|                          | Starch with Enzyme       | -55.08396* | 2.39484 | .000  | -63.3753 | -46.7927 |
|                          | Starch without Enzyme    | .00000     | 2.39484 | 1.000 | -8.2913  | 8.2913   |
|                          | Cellulose with Enzyme    | -31.40835* | 2.39484 | .000  | -39.6997 | -23.1171 |
|                          | Total sago effluent      | -21.25315* | 2.39484 | .000  | -29.5445 | -12.9619 |
| Total sago effluent      | Glucose                  | -58.59065* | 2.39484 | .000  | -66.8820 | -50.2994 |
|                          | Maltose                  | 21.25315*  | 2.39484 | .000  | 12.9619  | 29.5445  |
|                          | Sucrose                  | 14.81618*  | 2.39484 | .000  | 6.5249   | 23.1075  |
|                          | Starch with Enzyme       | -33.83081* | 2.39484 | .000  | -42.1221 | -25.5395 |

**Table 12** continued

|                                |            |         |      |          |         |
|--------------------------------|------------|---------|------|----------|---------|
| Starch without<br>Enzyme       | 21.25315*  | 2.39484 | .000 | 12.9619  | 29.5445 |
| Cellulose with<br>Enzyme       | -10.15520* | 2.39484 | .011 | -18.4465 | -1.8639 |
| Cellulose<br>without<br>Enzyme | 21.25315*  | 2.39484 | .000 | 12.9619  | 29.5445 |

\*. The mean difference is significant at the 0.05 level.