SEQUENTIAL SACCHARIFICATION AND SIMULTANEOUS FERMENTATION (SSSF) OF SAGO “HAMPAS” FOR THE PRODUCTION OF BIOETHANOL

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DECLARATION

I hereby declare that no portion of work referred in this project entitled “Sequential Saccharification and Simultaneous Fermentation (SSSF) of Sago “Hampas” for the Production of Bioethanol” has been submitted in support of an application for degree qualification of this or any other university or institution of higher learning.

_______________________
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<tr>
<td>BOD</td>
<td>Biochemical Oxygen Demand</td>
</tr>
<tr>
<td>CH$_3$CH$_2$OH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Double Distilled Water</td>
</tr>
<tr>
<td>DNS</td>
<td>Dinitrosalicylic</td>
</tr>
<tr>
<td>FPU</td>
<td>Filter Paper Unit</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure Applied Chemistry</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>m</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation per Minute</td>
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<tr>
<td>SHF</td>
<td>Separate Hydrolysis and Fermentation</td>
</tr>
<tr>
<td>SSSF</td>
<td>Simultaneous Saccharification Fermentation</td>
</tr>
<tr>
<td>SSSSF</td>
<td>Sequential Saccharification and Simultaneous Fermentation</td>
</tr>
<tr>
<td>v</td>
<td>Volume</td>
</tr>
<tr>
<td>w</td>
<td>Weight</td>
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Sequential Saccharification and Simultaneous Fermentation (SSSF) of Sago “Hampas” for the Production of Bioethanol.

Berry Rence Anak Senawi (25990)

A final report submitted in partial fulfilment of the Final Year Project 2 (STF 3015) Resources Biotechnology course.

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ABSTRACT

Bioethanol is one of the sustainable and renewable energy sources to replace fossil fuel. In this study, bioethanol was produced from sago hampas by using *Saccharomyces cerevisiae*. Sequential saccharification and simultaneous fermentation (SSSF), a modified version of simultaneous saccharification and fermentation (SSF) was performed on the sago hampas in different concentration which is 2.5% (w/v) and 5.0% (w/v) for five days. The samples taken from fermentation were analysed via DNS reducing sugar assay, phenol-sulphuric total carbohydrate assay and High Performance Liquid Chromatography (HPLC). This study has shown that ethanol was able to be produce from sago hampas using *S. cerevisiae* via SSSF. From the results obtained, 5.0% sago hampas give the highest ethanol yield which is 77.43% with a significant ratio of lactic acid and acetic acid.

Keywords: Bioethanol, DNS, High performance Liquid Chromatography, Phenol-Sulphuric, Sequential Saccharification and Simultaneous Fermentation, *Saccharomyces cerevisiae*.

ABSTRAK

Bioetanol adalah salah satu sumber tenaga yang mampu dan boleh diperbaharui untuk menggantikan bahan api fosil. Dalam kajian ini, kami menghasilkan bioetanol daripada hampas sagu dengan menggunakan *Saccharomyces cerevisiae*. Teknik sakrifikasi berturut dan penapaian serentak iaitu versi yang diubah suai daripada SSF telah dilakukan ke atas sagu hampas dalam kepekatan yang berbeza iaitu 2.5% (w/v) dan 5.0% (w/v) selama lima hari. Sampel yang diambil daripada penapaian dianalisis melalui ujian DNS, fenol sulfurik dan Kromatografi Cecair Berprestasi Tinggi (KCBT). Kajian ini telah menunjukkan bahawa etanol adalah dapat dihasilkan daripada hampas sagu menggunakan *S. cerevisiae* melalui SSSF. Keputusan analisa yang diperolehi daripada kajian ini menunjukkan bahawa 5.0%(w/v) hampas sagu telah menghasilkan etanol tertinggi iaitu sebanyak 77.43% dengan nisbah asid laktik dan asid asetik yang sesuai.

Kata Kunci: Bioetanol, DNS, Kromatografi Cecair Berprestasi Tinggi (KCBT), Fenol-Sulfurik, Sakrifikasi Berturut dan Penapaian Serentak, *Saccharomyces cerevisiae*.
CHAPTER 1
INTRODUCTION

1.1 Background Study

In recent years, environmental problems due to the over exploitation of fossil fuels have escalated. Fossil fuels that are widely used in the transportation sector release greenhouse and other dangerous gases into the environment. Therefore, new alternative sources of energy such as bioethanol and biodiesel are introduced.

Bioethanol is a promising substitute to fossil fuels due to its carbon dioxide neutrality and sustainability (Cervero et al., 2010). According to the literature, bioethanol release zero carbon dioxide output into the atmosphere as it is recycled through photosynthesis (Ohgren et al., 2006; Araque et al., 2008; Yamashita et al., 2010). Furthermore, when compared to fossil fuels, bioethanol burns completely and cleanly, produce only water and carbon dioxide.

Presently, bioethanol is produced by the fermentation of carbohydrate using fermenting organisms such as *Saccharomyces cerevisiae, Zymomonas mobilis and Escherichia coli* (Vincent et al., 2011 b). Most bioethanol is produced from the fermentation of sugar cane, cane juice, corn starch, grains, and potato starch (Cervero et al., 2010). This is called the first generation bioethanol and its production uses crop-based raw materials. However, as a result of growing demands for bioethanol, high cost of raw materials and the competition with crop-products, the search for alternative sources of fermentable carbohydrate such as lignocellulosic biomass was initiated. Lignocellulosic biomass is an abundant source of carbohydrate such as cellulose, hemicellulose and can be found in agricultural waste, forest waste and food-based industrial waste such as sago “hampas” (Cervero et al., 2010).
In this study, sago “hampas” was used to produce bioethanol. Sago “hampas” is an ideal feedstock as this material contains high lignocellulosic content. Furthermore, sago “hampas” from sago starch processing industries from Mukah and Sibu division of Sarawak (Adeni et al. 2010) is abundantly and is readily available for exploitation. According to a study done by Bujang et al. (1996), it is estimated that 7 tons (t) of sago “hampas” are produced daily from a single sago starch processing mill. This sago “hampas” is usually washed off into the nearby stream together with waste water which leads to serious environmental issues since it has high chemical oxygen demand (COD) and biological oxygen demand (BOD) (Adeni et al., 2010). Sago “hampas” contains high starch concentration of up to 66% and 16% fibre per weight basis (Chew and Shim, 1993), which are suitable raw materials for ethanol production. To produce bioethanol from sago “hampas”, this material was first converted to glucose through enzymatic hydrolysis reactions using amylase and cellulase. The glucose produced from the reaction was then converted in-situ using *S. cerevisiae* into ethanol via fermentation. This procedure is called Simultaneous Saccharification and Fermentation (SSF) method and it is currently a favoured process as this method is more profitable and time saving.

In this study, we modified the current SSF procedure to a sequential process which is referred as Sequential Saccharification and Simultaneous Fermentation (SSSF) process to further reduce the organic load in sago “hampas” and ultimately produced more bioethanol. The term sequential refer to a two steps process of enzymatic hydrolysis of starch, followed by the saccharification of cellulose in a same vessel.
1.2 Problem Statement

The production of the first generation bioethanol cannot meet the demand of our transportation sector as its production cost is very uneconomical. Thus, second generation bioethanol was introduced by utilizing lignocellulosic biomass and agricultural waste to save cost, to be more profitable and to meet the increasing world-wide demand for bioethanol. In this study, sago “hampas” was utilized to produce ethanol through Sequential Saccharification and Simultaneous Fermentation (SSSF) using amylase, cellulase and *S. cerevisiae*.

Most of the previous studies on bioethanol production from sago “hampas” only utilize the starch portion of the “hampas” while the remaining solid residue is discarded, while other studies have been done on the bioconversion of only the solid residue. Therefore, in this study, our SSSF was used to produce ethanol through a sequential process in which the starch of the sago “hampas” is first utilized followed by the solid residue. This was achieved by using amylase and cellulase enzyme which function to break down complex sugar into simple sugar, and *S. cerevisiae* as a fermenting microorganism.

1.3 Objectives

The objectives of this study are:-

1. To produce bioethanol from sago “hampas” through Sequential Saccharification and Simultaneous Fermentation (SSSF) using amylase, cellulase and *S. cerevisiae*.
2. To investigate the feasibility of increasing bioethanol production via Sequential Saccharification and Simultaneous Fermentation (SSSF) of sago “hampas”.
3. To determine the total reduction in sago “hampas” composition after the SSSF processes.
CHAPTER 2

LITERATURE REVIEW

2.1 Bioethanol

Bioethanol is ethanol that is produced through the fermentation of biomass. Ethanol, also known as ethyl alcohol is defined as a clear colourless liquid that is biodegradable, and environmentally friendly biofuel. It is now known as a promising substitute to fossil fuels. Ethanol can be classed as exotic synthetic oxygen containing organic chemicals because of its unique combination of properties as a solvents, germicides, antifreezes, fuels, depressants, and chemical intermediate for other chemical due to its versatility properties (Ingledew, 1999; Favela et al., 2003; Pramanik, 2003; Pramanik, 2005).

The complete chemical formula for ethanol is $\text{CH}_3\text{CH}_2\text{OH}$. Ethanol consists of two components which are an ethyl group ($\text{CH}_3\text{CH}_2-$) and an alcohol group (-OH). This hydrocarbon burn completely producing water molecules and carbon dioxide which is used directly for photosynthesis (Ohgren et al., 2006; Araque et al., 2008; Yamashita et al., 2010), resulting in zero net carbon released and water molecule. Ethanol is also a high octane fuel which has replaced lead while enhancing petrol performances (Altintas et al., 2002). It is a biofuel that can be renewed and produce from crop-based raw material such as corn and sugar cane and lignocellulosic biomass, through the fermentation of carbohydrates using fermenting microorganisms such as $\textit{Saccharomyces cerevisiae}$, $\textit{Zymomonas mobilis}$ and $\textit{Escherichia coli}$ (K011)(Vincent et al., 2011 b).

In addition to fermentation, ethanol can also be produced chemically by forcing ethylene to react with steam. However, this method is not efficient, high in production cost and low in productivity, to meet the global demands for fuel ethanol.
2.2 Lignocellulosic Biomass

Lignocellulosic biomass is a non-digestible substance which content mainly cellulose, hemicellulose and lignin. These biological materials are readily available for exploitation as a potential food, fuels and chemical feed stocks (Grahmann and Himmel, 1991). Lignocellulose can be found in many sources such as wood, grass, grains, sago “hampas”, agricultural waste and other indigestible plants (Zhang et al., 2009). Recently, lignocellulose has been recognised as a raw material for second generation bioethanol production because it does not compete with foodstock such as corn and sugar cane which is used in the production of first generation bioethanol. Furthermore, lignocellulose is relatively low cost (Badal et al., 2005), because it can be found from many sources.

2.3 Sago “hampas” and its compositions

Sago, also, known by its scientific name *Metroxylon sagu*, is from the genus *Metroxylon* and belongs to the Palmae group. Sago “hampas” is a lignocellulosic by-product from sago processing factory after starch extraction.

According to a study done by Bujang et al. (1996), there are three types of sago waste which are woody bark, wastewater and starchy fibrous sago pith residue or “hampas”. This “hampas” is usually washed off into the waterway together with waste water (Cecil et al. 1982), which leads to serious environmental problems (Khan and Husaini, 2006).

Studies done by previous researchers showed that there are differences in sago “hampas” composition due to the quality of the extraction processes (Adeni et al. 2010). For example, a study done by Chew and Shim (1993), showed that sago “hampas” contain 66% starch, 15% fibre and 1% protein while another study done by Horigome et al., (1991), showed that sago “hampas” contain 73% of starch, 13% fibre 1% protein.
Due to the high content of starch and fibre, sago “hampas” has been recognised as a raw material for the production of ethanol to supplement and replace crop-based raw materials which is used for the production of first generation of bioethanol (Chew and Shim, 1993). Moreover, sago “hampas” is also relatively cheaper compared to the conventional crop-based raw materials.

2.4 Cellulase

Cellulose is a major component in all lignocellulosic biomass including sago “hampas”. Cellulose can be hydrolysed to its simple glucose molecule by a group of enzymes called cellulases. Cellulase is an enzyme that contain at least three groups of enzyme which are endoglucanase, exoglucanase and β-glucosidases. Cellulases hydrolyse cellulose into D-glucose by breaking the 1,4-β-D-glycosidic linkages in cellulose. D-glucose produced from the reaction can then be used to produce ethanol through fermentation using the yeast *S. cerevisiae* (Krisna et al., 2001).

2.5 Amylase

Amylase is an enzyme that catalyses the breakdown of starch into glucose. Amylases are glycoside hydrolyses that act on the α-1-4-glycosidic linkages production α-glucose (Prasanna, 2005). The glucose produced from the reaction can then be converted into ethanol by *S. cerevisiae*. This enzyme will be used in the first stage of this study since sago “hampas” contain high starch percentage.
2.6 *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* is a eukaryotic microbe that is globular shaped, yellow-green yeast and belongs to the fungi kingdom (Landry et al., 2006). *S. cerevisiae* cell wall is made of chitin with no peptidoglycan and its lipids is linked by ester bond. It is considered as yeast because it is unicellular and it cannot form a fruiting body like other fungi does. This yeast has both asexual and sexual reproduction option. According to Madigan et al. (2006), *S. cerevisiae* is able to produce ethanol from glucose through both aerobic and anaerobic fermentation. They are also able to survive low oxygen concentration for an extended period.

*S. cerevisiae* is widely used in the food and beverages industry. It is used in the production of ethanol in the alcoholic beverages and also in the bread making. Recently, *S. cerevisiae* is used extensively in batch fermentation to convert glucose into ethanol for the production of biofuels as it is capable of rapid glycolysis under optimal conditions, producing more than 50 mol of ethanol per h per g of cell protein (Casey et al., 1986).

Figure 1. *Saccharomyces cerevisiae* culture at 24 hours.
2.7 Sequential Saccharification and Simultaneous Fermentation (SSSF)

Sequential Saccharification and Simultaneous Fermentation (SSSF) is a modified version of the currently used Simultaneous Saccharification and Fermentation (SSF). SSF is a method that is primarily used for the production of ethanol from lignocellulosic biomass. In SSF, enzymatic saccharification of cellulose by cellulolytic enzymes and fermentation of the sugar produced by the reaction using *S. cerevisiae* occurs at the same time and in the same vessel. This method has been studied for several decades and shows great potential for mass production of bioethanol (Takagi et al., 1977; Asli et al., 2008). A study done by Dowe et al. (2008) reported that SSF method is a preferred method to produced ethanol from lignocellulosic biomass. According to Falbe et al. (2005), SSF method is proven to be far superior to Separate Hydrolysis and Fermentation (SHF) method in terms of overall ethanol production.

The benefits of performing the enzymatic hydrolysis together with the fermentation instead of separating them are to reduce the end-product inhibition of enzymatic hydrolysis and to reduce the investment cost. Another advantage of using SSF is the reduction of potential microbial contamination with the presence of ethanol in the culture medium (Wu et al., 1998).

SSSF is a two-stage process which separate the hydrolysis of starch and cellulose. In this study, SSSF is performed by introducing amylase enzyme into the fermentation media to digest the starch on the first day of the fermentation. The fermentation media was left for 3 days to allow the reaction of amylase and starch. On the third day of the fermentation, cellulase was introduced to digest the remaining cellulose and the fermentation was continued until the fifth days of the fermentation. The enzyme amylase is used in the first stage of the SSSF while cellulase is used in the second stage. SSSF was performed to digest the
remaining solid remnant after the starch is hydrolysed by amylase and this will help to increase the ethanol yield.

2.8 Reducing Sugar Assay (Dinitrosalicylic Acid Assay)

Dinitrosalicylic Acid Assay (DNS) is a method that is usually used for reducing sugar assay. DNS is recommended by the IUPAC commission on biotechnology for measuring standard cellulase activities against filter paper. This assay is also applicable to measure the amount of amylase, pectinases, xyloglucanases, and xylanases activities.
2.9 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) as shown in Figure 2 below is a highly improved form of column chromatography. It is used mainly for the analysis of samples to separate and detect additives and contaminants in samples. In this method, complex mixtures are broken down into individual compounds on the base of their polarity which will be identified and quantified by detectors and data handling (Angelika et al., 2001). Other than that, HPLC also function to purify and quantify the compounds in the samples.

Figure 2. High Performance Liquid Chromatography (HPLC) System.
2.10 Filter Paper Unit (FPU)

According to Adney and Baker, (2008), Filter Paper Unit (FPU) assay is a specific enzyme activity assay that is performed using the protocol described by the official National Renewable Energy Laboratory (NREL) procedure. This method is recommended by the International Union of Pure and Applied Chemistry (IUPAC) as a guideline to determine the cellulase activity in terms of “filter-paper units” (FPU) per millilitre (FPU/mL) of an original enzyme solution as stated by Ghose (1987). In this method, the cleavage of the glycosidic bond is detected by the parallel and identical treatment of three categories of experimental tubes which consist of assay mixtures, blanks and controls, and glucose standard. The substrate used is a 50 mg Whatman No. 1 filter paper strip (1.0 x 6.0 cm) (NREL, 2008).
CHAPTER 3
MATERIALS AND METHODOLOGY

3.1 Materials

The materials used in this study were:

1. Sago "hampas"
2. *Saccharomyces cerevisiae* (ATCC 24859)
3. Cellulase enzyme (Accelerase 1500, Genencor, United States)
4. Amylase enzyme (ANG, Novoenzyme)
5. 10X Yeast Peptone solution
   i. 10 g yeast
   ii. 20 g peptone
   iii. 1000 ml citrate buffer
6. DNS Reagent
   i. 1 g Dinitrosalicylic acid
   ii. 200 mg crystalline phenol
   iii. 50 mg sodium sulphite
   iv. 100 ml of 1% NaOH
7. Phenol 5%
8. Sulphuric Acid 96%
9. 1 M Citrate buffer
10. Standard Glucose Stock (10 mg/ml)
3.2 Methodology

3.2.1 Cellulase Activity Assay Filter Paper Unit (FPU)

The cellulose activity assay was performed according to the International Union of Pure
Applied Chemistry (IUPAC).

3.2.1.1 Enzyme Assay

Filter paper of pre-determined sized of 1 X 6 cm were placed into each 13 X 100 test

tubes. 1.0 ml of 0.05 M buffer, pH 4.8 was added into the tubes to saturate the filter paper
strip. The test tubes were then equilibrated to 50 °C. After that, 0.5 ml of enzyme diluted will
be added appropriately in citrate buffer. Two dilutions were made of each enzyme sample in
which one dilution releasing more than 2.0 mg of glucose (absolute amount) and the other
dilution releasing below 2.0 mg of glucose. This step was followed by incubation at 50 °C for
exactly 60 minutes. After the incubation period, each of the assay tube was removed from 50
°C bath and the enzyme reaction was stopped by adding 3.0 ml of DNS reagent right after the
tubes were removed and mixed well.

3.2.1.2 Blank and Controls

Reagents blank : 1.5 ml citrate buffer

Enzyme control : 1.0 ml citrate buffer + 0.5 ml enzyme dilution (A separate control

was prepared for each dilution tested)

Substrate control : 1.5 ml citrate buffer + filter paper strip