Faculty of Resource Science and Technology

Effect of Peptone Supplementation on Bioethanol Fermentation using Sago Frond Sap

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A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
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Programme of Resource Biotechnology
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ABSTRACT

Utilization of lignocellulosic biomass is preferable to yield bioethanol since it is easily available and able to minimize environmental pollution. However, lignocellulosic biomass including sago starch, sago '*hampas*' or sago in form of hydrolysate involving high cost of production due to need to undergo pre-treatment. Thus, to minimize the cost needed, potential of sago frond sap as a substrate are currently being studied. Related to our study, it is not only just focusing on the potential of sago frond sap for yeast growth but the impact of adding peptone towards yeast growth and bioethanol production were also being highlighted. Technically, yeast required nitrogen source such as peptone to grow as well as yielding ethanol. Batch fermentation was performed by exposing yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) with two types of fermentation media consisting of 100 mL sago frond sap only and other media uses similar amount of substrate but being supplemented with 5 g/L peptone. During fermentation, the temperature was retained at 30ºC with a pH of 5.5. Based on the result, *S. cerevisiae* able to grow in sago frond sap producing ethanol. In fermentation media supplemented with 5 g/L peptone, both *S. cerevisiae* growth (9.1 g/L) and ethanol production (11.01 g/L) was enhanced compared to control media (Dry Cell Weight, DCW - 3.9 g/L, Ethanol production - 4.36 g/L). It is due to peptone provide diverse form of nutrients essential for yeast growth as well as ethanol production. To conclude, sago frond sap can become feedstock of interest which is relevant to be used in bioethanol industry. Also, presence of peptone is required to induce higher yield of yeast biomass as well as ethanol.

Key words: Bioethanol, Sago frond sap, Batch fermentation, *Saccharomyces cerevisiae*, Peptone.

ABSTRAK

Penggunaan biojisim lignoselulosa adalah lebih baik untuk menghasilkan bioethanol kerana mudah didapati dan boleh mengurangkan pencemaran alam sekitar. Walau bagaimanapun, biojisim lignoselulosa termasuklah pati sagu, hampas sagu atau sagu dalam bentuk hidrolisat melibatkan kos penghasilan yang tinggi kerana ia memerlukan pra-rawatan. Oleh itu, untuk meminimumkan kos yang diperlukan, potensi getah pelepah sagu sebagai substrat sedang di kaji. Berkaitan dengan kajian, ia bukan sahaja memberi tumpuan kepada potensi getah pelepah sagu untuk pertumbuhan yis tetapi kesan penambahan pepton terhadap pertumbuhan yis dan penghasilan bioethanol turut diketengahkan. Secara teknikal, yis memerlukan sumber nitrogen untuk tumbuh serta menghasilkan etanol. Penapaian kelompok dilakukan dengan mendedahkan yis, Saccharomyces cerevisiae (S. cerevisiae) *dengan dua jenis media penapaian yang terdiri daipada 100 mL getah pelepah sagu sahaja dan media lain yang menggunakan jumlah substrat yang sama tetapi di tambah dengan 5 g/L pepton. Semasa penapaian, suhu dikekalkan pada 30ºC dengan pH 5.5. Berdasarkan keputusan tersebut, S. cerevisiae mampu tumbuh dalam getah pelepah sagu yang menghasilkan etanol. Dalam media penapaian yang di tambah dengan 5 g/L pepton, pertumbuhan yis (9.1 g/L) dan penghasilan etanol (11.01 g/L) telah dipertingkatkan berbanding media kawalan (Berat sel kering – 3.9 g/L, penghasilan etanol – 4.36 g/L). Hal ini kerana pepton membekalkan pelbagai bentuk nutrien penting untuk pertumbuhan yis serta penghasilan etanol. Kesimpulannya, getah pelepah sagu boleh menjadi bahan mentah yang menarik yang relevan untuk digunakan dalam industri bioetanol. Kehadiran pepton turut diperlukan untuk mendorong hasil biojisim yis dan etanol yang tinggi.*

***Kata kunci**: Bioetanol, Getah pelepah sagu, Penapaian kelompok,* Saccharomyces cerevisiae, *Pepton*

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LIST OF ABBREVIATIONS

$	Dollar
%	Percent
% (v/v)	Percent of volume of solution in a total volume of a solution
µm	Micrometre
ATP	Adenosine Triphosphate
DCW	Dry cell weight
g	Gram
g/g	Gram per gram
g/L	Gram per litre
g/L/h	Gram per Litre per hour
H_2S	Hydrogen Sulphide
H_2SO_4	Sulphuric acid
HPLC	High Performance Liquid Chromatography
kg	Kilogram
M	Molarity
m^3	Meter cube
mL	Millilitre
mm	Millimetre
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
ºC	Degree Celsius
ºF	Fahrenheit
PDA	Potato Dextrose Agar

RID	Refractive Index Detector
rpm	Rotation per minute
S. cerevisiae	*Saccharomyces cerevisiae*
USA	United States of America
YMB	Yeast Malt Broth
YMPG	Yeast Growth Liquid Medium
Z. mobilis	*Zymomonas mobilis*

CHAPTER 1

INTRODUCTION

1.1 Research Background

Sources of energy indeed becomes a must for the development of rapid global population, accelerating urbanisation, demographic and social of a particular country or region (Hall *et al.*, 2013). Boosting energy demand is necessary to invent advanced technologies. This is because the progression of industrial sector is greatly influenced by the energy utilisation. According to Zakaria *et al.* (2021), the main problem of the conventional energy production in Malaysia is heavily rely on fossil fuel. Shafie *et al.* (2011) mentioned that 94.5% of the electric generated in Malaysia comes from fossil fuels during 2009. Rashidi *et al.* (2021) further stated that 83.2% of fossil fuel is utilised for electricity production in 2017. This indicates that there is continuation of the fossil fuel dependency.

Therefore, to reduce the dependency towards the fossil fuel, finding an alternative energy that is environmentally friendly is crucial to substitute fossil fuel. Many countries developed enormous interest in generating a renewable energy which is bioethanol. China, United States of America (USA), India, Brazil, Canada and European Union are actively producing bioethanol. Edeh (2020) stated that USA uses corn glucose, yielding bioethanol approximately at 57.7 billion litres in 2016. For Brazil, it utilises sugarcane sucrose, producing bioethanol at 27.6 billion litres. Basically, bioethanol can be produced via converting lignocellulosic biomass which consists of sugars or starch (Nguyen *et al.*, 2016). It is done by fermenting the available monomeric sugar derived from carbohydrate sources using microbes (Edeh, 2020).

Generally, *Saccharomyces cerevisiae* (*S. cerevisiae*) is the common microorganism used in fermentation process. It is being chosen since it is the simplest form eukaryotic organism (Parapouli *et al.*, (2020). Consequently, it allows scientists to make use of *S. cerevisiae* since it is easy to be cultured (Salari & Salari, 2017). The unique regarding the *S. cerevisiae* is to produce ethanol and grow aerobically or anaerobically. The state of fermenting ethanol depends on the sugar utilized by it. At the final stage of ethanol fermentation, carbon dioxide and ethanol will be generated. *S. cerevisiae* has the capability of shifting the respiratory metabolism from aerobic to the anaerobic specifically fermentative mode in absence of oxygen. The shift of metabolism is generally known as Pasteur Effect (Ariff *et al*., 2000).

Supplementation of nitrogen sources is crucial because it has been proven that the addition of nitrogen source increases the yeast cell biomass as well as bioethanol produced. However, not all nitrogen sources can do so due to differences in their composition. Types of nitrogen used may also influence the cell biomass yield. A previous study conducted by Zohri *et al*. (2017) involved yeast is grown in Yeast Growth Liquid Medium (YMPG) using batch fermentation mode. He and his colleagues found that higher yeast cell biomass is produced (152.9 g/L) and ethanol generated (2.1% v/v) using 1.5 g of organic nitrogen, urea. It is higher compared to the yeast biomass supplemented inorganic nitrogen, 3.29 g/L of ammonium sulphate, and 2 g/L of ammonium nitrate which are 141.9 g/L and 142.4 g/L respectively. Therefore, organic nitrogen would be suitable to enhance the yeast cell biomass because of exhibiting complete nutrients.

In this study, effect of peptone supplementation was examined on the *Saccharomyces cerevisiae*'s bioethanol fermentation. The substrate used in this study is sap extracted from sago frond. This is due to sago frond have longer degradation rate that promoting environmental pollution. Thus, it become biomass interests due to its potential in bioethanol industry. Sago frond is a type of lignocellulosic biomass which exhibit low-cost production (Awg-Adeni *et al.*, 2018). Matnin *et al.* (2021) mentioned that the high potential of frond sap used as substrate as it consists of free sugar content. This indicates that the sap can be directly used in which the pre-treatment and modification of it is negligible. During fermentation, 5 g/L peptone was added to fermentation to study its effect on yeast biomass as well as ethanol production. The significant of peptone addition is lack of nitrogen in palm sap tends to affect the yeast growth for bioethanol fermentation. The yeast cell biomass was obtained from its dry cell weight (DCW) measurement. Its product, ethanol that has been produced and its carbon source, glucose consumed was determined by performing High Performance Liquid Chromatography (HPLC).

1.2 Objectives

The objective of this study is:

1) To determine the effect of peptone on the ethanol production using sago frond sap as an alternative substrate.
2) To analyze the correlation of yeast growth and bioethanol production when yeast utilize glucose in sago frond sap.

CHAPTER 2

LITREATURE REVIEW

2.1 Bioethanol

2.1.1 Definition of Bioethanol

Bioethanol or commonly known as Ethyl alcohol with its chemical composition that can be written as C_2H_5OH. Edeh (2020) mentioned that it is a type of colourless liquid that consists of 35% oxygen obtained from fermentation using carbohydrate source derived from sugarcane, corn, soybean. Presence of oxygen makes it more favourable due to it is being completely combusted. It capable of reducing the release of carbon dioxide gas to the atmosphere. Other than that, bioethanol also being combined with gasoline that aid in boosting the engine performance. According to Edeh (2020), this is due to bioethanol comprises high amount of octane and low boiling point which ensures engine effectivity.

2.1.2 Bioethanol Industry

Since fossil fuel consumption induces several environmental issues, global demand on bioethanol has shot up rapidly (Edeh, 2020). High demand of bioethanol can be proven in which majority of global biofuel generated and consumed hugely concentrated to bioethanol instead of biodiesel (Torroba, 2020). According to Torroba (2020), global utilisation of bioethanol in 2011 was 77.22 thousand m^3 which shot up to 115.39 thousand m^3 in 2019. This indicates that almost 49.43% of the bioethanol is greatly consumed by global population over 9 years. Since the trend of bioethanol consumption is relatively higher, larger amount of bioethanol has been produced to fulfil the global demand. In 2010, 85.83 thousand m^3 of bioethanol were generated which eventually increased to 109.68 thousand m^3 in 2018 (Torroba, 2020). However, in 2019, bioethanol demand exceeded its production.

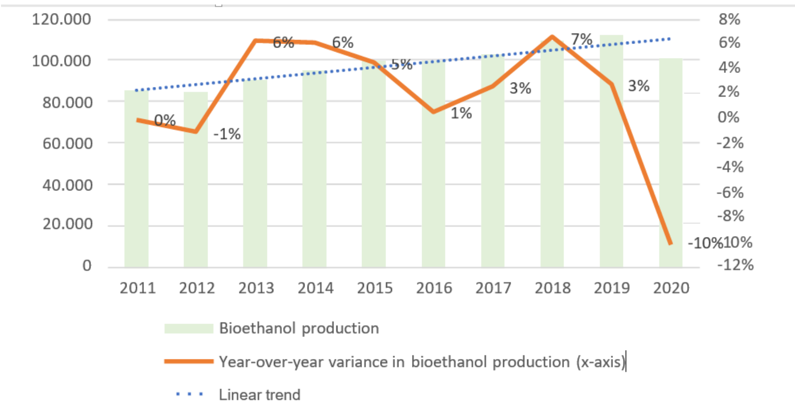

Figure 1: Global Bioethanol Production from 2011 to 2020. Adapted from *Liquid Biofuels atlas 2020-2021*, by Torroba, 2021, Inter-American Institute for Cooperation on Agriculture (IICA) 2021. Copyright [2021] by IICA Print Shop. Reprinted with Permission.

The downfall of bioethanol industry has been depicted in 2020 whereby both utilisation and production of bioethanol showed significant reduction. Only 97.75 thousand m^3 was utilised compared to 115.39 thousand m^3 in 2019 in which 15.29% significant reduction. Its generation also decreased from 112.62 thousand m^3 in 2019 to 101.25 thousand m^3. In 2020, USA and Brazil are the main country that consumed (49% and 29%) and generated bioethanol (52% and 32%) the most because they are the powerhouse in this industry. Downfall of bioethanol industry occurs due to it has been affected by pandemic COVID-19 that interferes the smooth flow of economy sector including Malaysia. Wahab (2021) reported that there was no bioethanol being exported from 2020 to 2021. There is a rapid reduction in international and domestic demand causing an economic slowdown. Thus, accelerating bioethanol industry must be done since industrial sector is heavily relies on it.

2.1.3 Bioethanol production

Amylase enzymes catalyse the hydrolysis of complex starch molecules to produce maltose and further yielding glucose. *S. cerevisiae* metabolize glucose by Embden-

Meyerhof Pathway or known as glycolysis to split glucose. At the end of the glycolysis step, 2 Pyruvate molecule associated with 2 ATP and 2 NADH molecule is produced. 2 Pyruvate molecules then enter alcoholic fermentation.

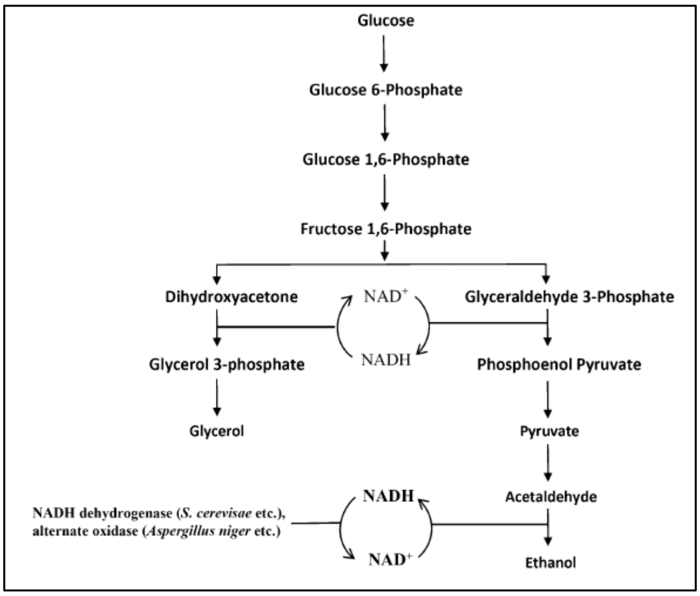

Figure 2. Fate of glucose during Glycolysis and fermentation pathway. Adapted from *Advances in Bioprocess Technology* (ed., pp. 165-199), by Kumar *et al.*, 2015, Chapter 10: Bioenergy: Biofuels Process Technology. Copyright [2015] by Pogaku Ravindra. Reprinted with permission.

During alcoholic fermentation, pyruvate is converted into ethanal, which is later further reduced to generate ethanol. During this phase, carbon dioxide is expelled. At certain times, carbon dioxide concentration increases. Rising ethanol gradually will kill the yeast cells when ethanol concentration is relatively high.

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

Figure 3. Overall equation of ethanol fermentation. Adapted from *Advances in Bioprocess Technology* (ed., pp. 165-199), by Kumar *et al.*, 2015, Chapter 10: Bioenergy: Biofuels Process Technology. Copyright [2015] by Pogaku Ravindra. Reprinted with permission.

Kumar *et al.* (2015) mentioned that 1 kg of glucose can yield the amount of ethanol at 0.51 kg and the rest is carbon dioxide in theory. Practically, less than 100% ethanol is produced since yeast utilizes ethanol for its growth. Compared to *S. cerevisiae*, *Zymomonas mobilis (Z. mobilis)* can produce a large amount of ethanol since it grows faster which is theoretically 97% (Kumar *et al.*, 2015: Todhanakasem *et al*., 2019). Only 90 to 93% ethanol is produced by *S. cerevisiae* fermentation. However, the usage of *Z. mobilis* limited to a certain substrate including glucose, fructose, and sucrose (Lin & Tanaka, 2006: Todhanakasem *et al.*, 2019). Starch cannot be utilized by *Z. mobilis* and other carbohydrate polymers as well. Thus, it is not suitable to be used in bioethanol industry that uses lignocellulosic biomass (Busic *et al.*, 2018). *S. cerevisiae* would be a suitable organism for fermentation since it can utilize diverse form of sugar which are glucose, sucrose, maltose, and starch as well during its growth (Salari & Salari, 2017).

2.2 Sago Palm

2.2.1 Morphology of Sago Palm

Sago palm or commonly known as *Metroxylon sagu* is a type of palm tree belongs to the family of Palmae. The word 'sago,' according to Flach (1997), originated from a Javanese language and literally refers to starch that inhabits the palm pith. Meanwhile, ‘Metroxylon’ can be defined as its xylem tissue consists of numerous parenchyma cells that primarily involved in water movement and starch storage. Sago palm able to grow naturally in mild environmental condition including freshwater swamp (Lim *et al.*, 2019). This is due to its great tolerance for saline conditions, which is advantageous to its growth. Apart from that, it also has the capability of growing in a tropical rainforest with warm temperature since it is extensively distributed in Southeast Asia region such as Malaysia, Indonesia, Thailand and Papua New Guinea (Johnson, 1977).

The maximum height of sago palm is approximately at 10 to 15 metres with its trunk diameter can be in 35 to 75 centimetres in terms of its length (Lim *et al.*, 2019). As for the stem of sago palm, it exists as straight, thick associates with absence of branch. It takes 3 years and a half for the sago palm forming its thick stem. The growing leaf can reach its maximum size at 6 to 9 metres (Sighal *et al.*, 2008). The leaf or frond formation can be observed approximately at 2 months in its life cycle. Flanch (1997) highlighted that harvesting the leaf can be performed when the sago palm reaches its age at 4 years old. Sago palm is classified as diecious plant because it reproduced asexually since its reproductive organ located on a different individual plant. It exhibits 2 types of life cycle which is haploid gametophyte and diploid sporophyte. Normally, the pollination takes place with the aid from wind that transfers the male pollen originated from torpedo cone into the female reproductive organ which less likely in form of cabbage.

The unique characteristics regarding the sago palm is it is the only palm that produce flower and fruit in a single time within their life cycle. Sighal *et al.* (2008) stated that it is classified as hapaxantic due to it undergone immediate mortality phase as soon as the flower or fruit is formed. Formation of fruit or flowers takes place when it achieves its maturity stage at 9-12 years. During maturity stage, a young sago palm tends to rapidly accumulate the starch found in trunk. This starch is obtained through the stored nutrients is being converted during its vegetative phase.

2.2.2 Application of Sago Palm

Nowadays, sago palm has become an economic value since different parts of it can be utilised as raw materials in diverse industrial sectors for the formation of valuable product (Sighal *et al.*, 2008). For instance, the sago starch is widely utilised in food, pharmaceutical, polymer, textile and others as well. Its frond or leaves can be make used to make papers since it can withstand to physical damage. This is because it consists of long fibres and large amount of cellulose. As for its '*hampas*', it can be used for feedstock feed typically for chicken and horses. Therefore, extensive on-going research indicates that discovering sago palm potential is crucial since it is limitless.

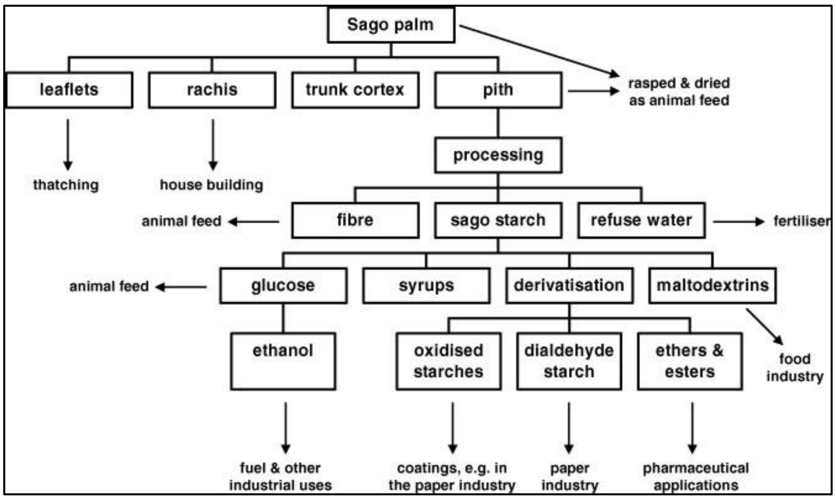

Figure 4: Application of Sago Palm. Adapted from *Carbohydrate Polymers 72*(1), (pp. 1-20), by Singhal *et al.* (2008). Industrial production, processing, and utilization of sago palm-derived products. Copyright [2007]. Reprinted with permission.

2.3 Sago Frond

Sago frond of the sago palm can be classified as long green cycad. This is due to the development of the frond originated from its trunk located at the centre of the palm tree. Diameter of the tree trunk influences the number of sago fronds produced. The higher the diameter of the sago palm tree trunk, the larger the number of sago frond produced. Basically, Flach (1997) mentioned that a healthy sago palm tends to generate 24 fronds provided it thrive at optimal temperature. It grows optimally at 25ºC and above associated with the higher relative humidity which is at 70% (Sighal *et al.*, 2018).

Besides, exposing sago palm to warm environment is crucial as it prefers to receive indirect light to perform photosynthesis. According to Flach (1997), he further stated that the formation of sago frond takes place every single month by replacing the old, dead sago frond. A single frond able to grow approximately at 5 to 8 metres in terms of its length. Previous study conducted by Sunarti *et al.* (2019) mentioned that there are two important components presents in sago frond which are crude fibre and carbohydrate. Crude fibres mainly consist of 23.70% cellulose, 26.17% hemicellulose and 29.7% lignin. It is relatively lower than other lignocellulosic biomass including pineapple and banana stem fibres due to different of types of sources. As for carbohydrate parts, it has higher carbohydrate portion compared to cellulose since it has 26.38% of it.

Due to it takes such a long period to degrade sago frond, an alternative was developed in order to minimise pollution (Matnin *et al.*, 2019). It has been widely used for numerous purposes including papermaking, producing bag and mats. Besides, it also crucial for generating sago frond sap. Ahmad *et al.* (2016) stated that a type of prebiotic sugar, cellobiose can be produced through enzymatic hydrolysis process due to the presence of cellulose component in fronds.

2.4 Frond Sap

Frond sap is a type of fluid that occupies within sago frond. The volume of sap extracted is greatly depends on several factors. There are two factors that influences the sap volume. This includes moisture content and size of sago frond that highly affected by the age of the sago palm itself (Flach, 1997). Kosugi *et al.* (2010) mentioned that high moisture content associated with large size of the sago fronds tends to generate large amount of sap