ETHANOL FERMENTATION FROM YEAST ON SAGO MILK

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List of Abbreviations

DCW	Dry cell weight
DE	Dextrose equivalent
DNS	3,5-dinitrosalicyclic acid
DS	Dry substrate
G	Gram
g/L	Gram per litre
hr	Hours
min	Minutes
OD	Optical density
Psi	Pounds (lb.) per square inch
rpm	Revolution per min
UV	Ultra violet
V	Working volume
v/v	Volume per volume
w/v	Weight per volume
^{0}C	Degree celsius
µm	Micrometer

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Ethanol Fermentation from Yeast on Sago Milk

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ABSTRACT

Sago milk samples were obtained from a factory at different times as a survey to determine the possibility of using sago milk for ethanol fermentation. Hydrolyzed sago milk (HSM), which acted as a substrate for the production of the ethanol was studied in a fermentation process using commercial Baker's yeast *Saccharomyces cerevisiae*. Upon analysis, all sago milk samples contained average of 94% of starch with dry matter ranging from 24% to 35% with average of 31%. In enzymatic hydrolysis, all samples of sago milk were convertible to reducing sugar at an average recovery of DE108%, much better than sago starch (20% DS) at recovery of DE 81.13%. The optimum concentrations of enzymes at 0.5 ul/L of Termamyl and 0.6 ul L of Dextrozyme were used in both hydrolysis of commercial sago starch and sago milk. When hydrolyzed sago milk was used as the substrate for batch ethanol fermentation, a sugar concentration of 100g/L produced 40.83g L ethanol with fermentation efficiency of 95.43%. Thus, hydrolyzed sago milk had high potential to become an alternative substrate to sago starch for ethanol production.

Key words: Sago starch, hydrolysis, *Saccharomyces cerevisiae*, ethanol fermentation

ABSTRAK

Susu sagu sampel diperoleh daripada kilang pada masa yang berlainan untuk menentukan kemungkinan mengunakan susu sagu untuk fermentasi etanol. Susu sagu yang dihidrolisis (HSM), digunakan sebagai substrak untuk menghasilkan etanol dengan mengunakan komersial "Baker's Yeast" atau Saccharomyces cerevisiae. Berdasarkan analisis, semua sampel susu sago dengan purata sebanyak 94% adalah kandungan kanji dan berat kering adalah dalam linkungan 24% kepada 35% dengan purata sebanyak 31%. Semua sampel susu sagu dapat memukarkan kanji kepada gula dengan purata kadar pemulihan sebanyak DE 108%, dan ini menunjukkan bahawa susu sagu lebih baik daripada kanji sagu (20% DS) yang mempunyai kadar pemulihan sebanyak DE 81.13% dalam hidrolisis proses. Kepekatan enzim untuk hidrolisis susu sagu adalah sama seperti yang dijalankan terhadap komersial kanji sagu iaitu 0.5 ug/L untuk Termamy 120L dan 0.6 ug L untuk Dextrozyme. Apabila susu sagu yang dihidrolisis digunakan untuk fermentasi etanol secara berkelompok, 100g/L kepekatan gula dapat menghasilkan etanol sebanyak 40.83g/L dengan effisen fermentasi sebanyak 95.43%. Dengan ini, hidrolisis susu sagu berpotensi untuk menjadi substrak alternatif kepada kanji sagu untuk penghasilan etanol

Kata kunci: Kanji sagu, hidrolisis, Saccharomyces cerevisiae, fermentasi etanol

1.0 Introduction

The Sago palm, scientifically known as *Metroxylon sagu*, is the oldest plant in Palmae Family and known at "Rumbia" by local people in Sarawak. It is the main staple and cash crop in Southeast Asia and Pacific apart from rice, corn, wheat and cassava. Due to its abundant availability of sago starch as cheap carbon source, it can potentially become alternative biofuel, foodstuff to prevent famine due to increase human population and manufacturing of food additives and non food products. It had been reported that about 60 millions tones of sago starch extracted from sago palms were produced per annum in South-east Asia (Wang *et al.*, 1996).

Sago milk is formed from the extraction of starch with water and the starch slurry pass through the series of centrifugal sieve to remove the fiber. The starch in sago milk is allowed to be precipitated in the sedimentation tank and water overflow before starch is removed and dried. Addition of water is important in extraction of the starch because it uses to dissolve and release the starch granule into sago milk. Sago flour, which is one of the sago palm derived products, is the main source of starch in Malaysia.

Furthermore, sago palms are among the starchy crops that generate the highest productivity of land area of 24t ha of starch per year compared rice (6t/ha), corn (5.5t/ha), wheat (5t/ha) or potato (2.5t ha) (Bujang & Ahmad, 2000). According to Flach (1983), he had reported mature sago palm weighed about 1,250 kg contain around 400 kg cortex and 850 kg pith. Pith contains starch that needs to be separated from the cellulosic cell walls of the trunk. The pith contains 425kg water, 175kg other material and 250kg starch that can be separated from the cellulosic cell walls of the trunk.

In this era of biotechnology, types of microorganisms have been utilized for various industrial biotechnologies including ethanol fermentation industrial. Fermentation is defined as anaerobic metabolic breakdown of a nutrient molecule such as glucose without net oxidation during fermentation which depends on what types of organism to yield lactate, acetic acid, ethanol or other reduced metabolites. According to Zakpaa *et al* (1998), fossil fuels were non-renewable energy source that were claimed to be exhausted in the next century. Ethanol fermentation could be one of the ways to replace fossil fuels and allowed reduction import of oil by developing countries and solid waste management (Doelle, 1994; Bujang *et al.*, 2000).

Batch fermentation represents growth in a closed system that contains a suitable growth supporting medium because this process requires all nutrients to be added before fermentation is initiated. Fermentation could determine the glucose consumption rate, biomass production rate and ethanol production rate. Ethanol, produced from renewable energy sources like biomass, brings the promising future in bio-fuel industry (Masrszalek & Kaminski, 2008). At present, it is used as an additive to petrol in fuel industry with the percentage of 10% ethanol and 90% gasoline.

Recently, higher production cost of sago starch for higher efficiency starch extraction and public acceptance of using food as source of biofuel have become the problem issues. Hence, sago milk can be used as alternative raw materials and change the perspective view of public because it have not undergo several process to produce food products. Furthermore, using food source for biofuel production had been criticized by NGO.

Sago starch cannot be directly fermented by yeast or bacteria due to its starchy materials. Therefore, starch must be hydrolyzed to simple sugars in order for the microorganisms to react on. Firstly, the sago starch had to be undergoing gelatinization before hydrolyzed to sugars by pretreatment acid or enzymes (Govindasamy *et al.*, 1992). The pH, amount of enzyme added and the incubation time during hydrolysis were controlled in order to see whether the sago milk was able to hydrolyze to yield highest possible of reducing sugar. Hence, the first part that needed to go through in this experiment was the gelatinization process because the amount of starch in the sago milk was estimated in the range of 24.0% to 35.4% based on dry matter of sago milk in this experiment.

1.1 Objectives

1) To determine the best concentration of sago milk to produce highest reducing sugar
2) To determine whether the reducing sugar from sago milk is able to be fermented
3) To determine whether dried sago starch (expensive) is able to replaced by sago milk (cheaper)
4) To explore whether the sago milk is able to reduce the cost production

2.0 Literature Review

2.1 Sago Palm

Sago palm has several advantageous characteristics which cause this plant to be highest starch producer and major cash crop plant in the Sarawak. One of the advantageous of sago palm is its reproduction without replanting because young palms will develop from these suckers and these suckers can be developed more before reaching its maturity at about 9 to 11 years (Bujang, 2006). Basically, suckers with the unopened buds are the best planting materials to develop into adult mature sago palm. Furthermore, this species can be grown in swampy area with minimum care and less serious pest attack.

All Metroxylon species except *Metroxylon sagu* are propagated by seeds. A fully grown up sago palm is about 10m to 12m high with a diameter at about 0.8m to 1m. As it is a hapaxanthic plant, it will flowers once in its lifetime before it dies. However, the plants will converts its nutrient into starch during vegetative stage and starch will accumulate in the trunk until the flowering stage (Aziz, 2002). In addition, the palm is immune to floods, drought, fire, strong wind and its large fibrous root system is able to remove pollutants and heavy metals which makes the sago excellence for sustainable agricultural due to concern of the environment. Furthermore, the sago forest can reduce the greenhouse effect. Biofuel can be one of the contributions to sustainable development to reduce the greenhouse gas emissions (Henke *et al.*, 2005; as cited in Prasad *et al.*, 2007).

2.2 Process of Sago Flour

Sago is a reliable source of carbohydrate and recently, the commercial production of sago flour in Malaysia mainly in Sarawak and small parts in Johor. This starchy crops have make Sarawak as one of the biggest exporters of sago (kamal *et al.*, 2007). The steps of production of sago flour mainly include debarking, rasping, sieving, washing, drying and packing (Bujang & Yusop, 2005). Firstly the sago trunk will be stripped off leaves and cut into 1m logs for easily handling and then will be transported to the sago mill. The hard and thick bark will be debarked by using debarking machine or manually by workers. Soon, the debarked log or sago pith will be mashed using a rasper followed by hammer mill before adding water to form starch slurries and drying it to form sago flour. Since, large amount of water is required to extract starch from fibers, the modern plant processing will be situated near the river for convenience method for waste disposal.

Sago starch is most probably preferable than cassava, sugar cane and maize as substrate to produce ethanol due to economic and geographical factors. However, starch yield depend on the condition of the soil and different from place to place (Bujang, 2006). According to Haryanto (1992, as cited in Bujang, 2006), the range of starch based on dry weight basis in Sarawak, Bengkalis, Jayapura and Maluku were 180kg to 385kg, 550kg, 250kg and 184kg respectively. Bujang *et al.* (1996) had reported that quantity of starch for each log that weighed between 100 to 130 kg was estimated to be about 20% of the fresh weight per log.

2.3 Sago Industries in Malaysia

In Malaysia, more than 90% of sago plantations were found in Sarawak where 75% of sago planting area in Mukah and more than 50% of the sago starch could be produced (Bujang & Ahmad, 2000, as cited in Bujang, 2006). This had shown that sago industry had the potential to grown in Sarawak where it grew commercially as a smallholder crop and exports 50, 000 tons of air-dried flour a year from the sago palm (Aziz, 2002). It had reported that sago starch had been ranking the fifth highest in agricultural revenue in Malaysia after pepper, palm oil, cocoa and rubber (Aziz, 2002). Furthermore, sago produces higher amount of starch at about 2 to 3 tons compare to cassava and maize which is 2 and 1 tons respectively (Singhal *et al.*, 2007). In addition, sago starch yield per unit area could be about 3 to 4 times higher than rice, corn, or wheat, while about 17 times higher than cassava (Karim *et. al*, 2008).

2.4 Uses of Sago Starch

Uses of sago starch-based-products have great potential in Malaysia market especially when there are improvements in product processing and quality. Sago starch has multitude of uses and it is a great abundant plant products besides a major source for energy. The potentials of sago industry in foodstuff include manufacture food additives, flavoring, monosodium glutamate, thickening soup and stabilizer for pharmaceutical products. In Malaysia, many food manufacturing industries have used sago flour as main ingredient in the production of vermicelli, noodle production, biscuits (tabaloi), cakes, bread, cracker, sago pearls, sago pudding and food syrup for non alcoholic drinks (Bujang & Ahmad,

1999). It also contributes to non food stuff such as production of papers, bioplastic, glue manufacture, animal feeds and mushroom cultivation (Ishizaki, 1997; Aziz, 2002).

2.5 Hydrolysis Process

Hydrolysis process is important in order to convert the sago starch into reducing sugars by pretreatment of the enzymes. This is because the microorganisms do not have the ability to digest the starch directly. Furthermore, glucose from the sago starch can be the substrate for both the ethanol and lactate fermentation to produce biofuel and bioplastic to overcome problem of fuel shortage and solid waste management (Bujang et al, 1998).

Bujang *et al.* (2000) had reported that conversion of sago starch to glucose during hydrolysis was at over 98% recovery. Two commercial enzymes Termamyl 120-L (thermostable alpha-amylase) and Dextrozyme 225/75L (mixture of glucoamylase and pullulanase) were used. Termamyl-120L, which the amylase could be originated from *Bacillus amyloliquefaceins*, *Bacillus licheniformis* or *Bacillus stearothermophilus*, was used to loosen the structure of the starch molecules by cleaving α-1,4 glucosidic bonds within the starch molecule and liquefied the starch suspension. Dextrozyme, which was made up of mixture glucoamylase from *Aspergillus niger* and pullulanase from *Bacillus acidopullulyticus*, used to saccharify the liquefied suspension to glucose (Rhee *et al.*, 1984).

The enzymatic activities of these enzymes as defined by Novo Nordisk were 120KNU/g and 225 AGU/ml respectively. Jolhery (2001) had reported that the optimum concentration

of Termamyl and Dextrozyme were at 0.5ul/g and 0.6ul/g respectively. Substrates, enzyme activity, reaction condition such as pH and temperature played important roles in these two step enzymatic hydrolysis processes (Sun *et al.*, 2002). Starch liquefaction processes were conducted in the presence of calcium to impart heat stability to the enzyme. The enzymatic conversion yield of starch into reducing sugar was being expressed as dextrose equivalent (DE), which defined as the percentage of reducing sugars present on dry solid basis.

2.6 Yeast

Saccharomyces cerevisiae or common name as Baker's yeasts, are traditional yeasts that have been used most commonly in batch fermentation. It metabolizes sugar rapidly under anaerobic condition and produces biofuel, beverage and carbon dioxide. Growth of the fermenting organisms might inhibit by high temperature and high substrate concentration due to osmotic stress (Jones *et al.*, 1981). *Saccharomyces cerevisiae* has the ability to cause the flocculants to adhere carbon dioxide and rise at the top of the fermentation vessel. The yeasts able to convert the 90% of all the glucose into ethanol and it's by products formed was mainly carbon dioxide under anaerobic condition.

It is also well known as old traditional yeast fermentation which has the advantageous of easily available, cheap, efficient anaerobic sugar metabolism, tolerates inhibitory industrial substrates better than other microorganisms, produces less biomass, higher protein, no glycerol by-products. Although *Saccharomyces cerevisiae* is unable to utilize xylose, but it can slowly metabolize xylulose, the ketoisomer of xylose.

2.7 Ethanol Production

Production of ethanol through fermentation process is one of the most essential processes in this world. This is because utilizing new raw material such as sago starch is easily available and it advantageous characteristics cause the production cost to be minimized. Since the natural energy resources such as fossil fuel, petroleum and coal have been estimated to last for a few more years, alternative energy source such as ethanol from fermentation process has gained more importance as fuel additive (Aziz, 2002).

As from the total recovery of the 98% using termamyl-120L and Dextrozyme in hydrolysis process (Bujang *et al.*, 2000), 30,500 tons of sago starch would produced 29,890 tons of glucose. Theoretically had shown that 1 mole of glucose produces 0.5 mole of ethanol which caused 29,890 tons of glucose could produce 14,945 tons of ethanol.

According to Jones *et al.* (1981), it had been reported that requirement nutrients for cell growth and maintenance were important in conducting fermentation process. Fermentation medium that contains NH_4Cl, $MgSO_4$, $CaCl_2$ and yeast extract can contribute to rapid cell growth and growth promoter (Cysewski & Wilke, 1976a,b). Today, ethanol production mostly done by the batch operation since the investment costs are low and do not require much control and low labour skill (Caylak & Vardar Sukan, 1998).

3.0 Materials and Methods

3.1 Materials

3.1.1 Sago starch

Commercial sago starch (SHM) was obtained in a supermarket available.

3.1.2 Sago milk

Fresh sago milk (1L) was obtained from a sedimentation tank at Herdsen Sago Sdn Bhd in Pusa.

3.1.3 Hydrolytic enzymes

Two types of enzymes (Novo Nordisk, 1990) were used namely; Termamyl-120L, (termostable α- amylase from *Bacillus licheniformis*, 120 KNU/g) and Dextrozyme (a mixture of glucoamylase from *Aspergillus niger* and pullulanase from *Bacillus acidopullulyticus*, 225 AGU/ ml).

3.1.4 Reducing sugar analysis reagents

Preparation of the reducing sugar reagents was based on Miller (1959).

1) Substrate solution: Standard solution of 100mg/ml concentration was prepared by dissolving 100 mg of glucose (ANALAR) in 100ml of distilled water.

2) 3,5 dinitrosalicylic acid (DNS) solution: Reagent was prepared by dissolving 1% of 3.5-DNS, 0.2% of phenol and 0.05% of sodium sulphite in 100 ml of 1% NaOH solution followed by stored in dark colored bottle.

3) Potassium sodium tartarate (Rochelle salt): 40 g of potassium sodium tartarate dissolved in distilled water and the volume was made up to 100ml.

3.1.5 Fermenting yeast

Dry baker's yeast (Fe Syn brand) or *Saccharomyces cerevisiae* at 10 g/L or 1 %, was used as inoculum (w/v), which purchased from a local supermarket.

3.1.6 Fermentation media

The fermentation media was 100 g/L reducing sugar from hydrolyzed sago milk, 10 g/L yeast extract (Difco, USA) and top up with distilled water to 1 Liter. The fermentor was autoclaved and cooled for overnight before fermentation initiated.

3.2 Methods

3.2.1 Preparation of sago milk

Dried sago starch (100g, 200g and 300g) were mixed with 1 Liter of distilled water respectively to act as control. Sago milk samples were obtained a sedimentation tank in the sago factory were preserved in the cold room before further analysis. The fresh sago milk was tested for dry matter, starch and reducing sugar analysis prior to enzymatic hydrolysis.

Figure 1: Sago milk from a sedimentation tank in Pusa

3.2.2 Hydrolysis of sago milk

The hydrolysis of the sago milk was based on hydrolysis of sago starch (Bujang *et al.*, 1999). The sago milk was filtrated before hydrolysis process and amount of filtrate would be recorded. The mixture was heated at 90°C to 100°C at 5 to 10 minutes for gelatinization. One Molar of NaOH and one Molar of H_2SO_4 were used to adjust pH to pH 6.5. Liquefaction was carried out by adding 30mg calcium ions (1kg of starch) which acted to stabilize the enzyme, followed by, 0.5ul of Termamyl 120L (per gram of starch) and

incubated for 2 hours. Amount of 0.6ul of Dextrozyme (per gram of starch) was added for saccharification process at pH 4.5 and incubated for 4 hours. At the end of the hydrolysis, 1ml of diluted sample was taken out to test for starch and reducing sugar analysis. Three replicates were done.

3.2.3 Batch fermentation on hydrolyzed sago milk

The medium was autoclaved at 121°C at 15 psi for 15 min. Upon cooling, dry baker's yeast (10 g/L) was added in batch fermentation study. Hydrolyzed sago milk was fermented by yeast to produce ethanol with basic parameters of temperature 34°C, pH 5.5 and agitation rate of 500 rpm. The pH was maintained automatically by addition of 1M NaOH or 1M H_2SO_4. The glucose, ethanol and DCW measurement were determined for 0 and 24 hours.

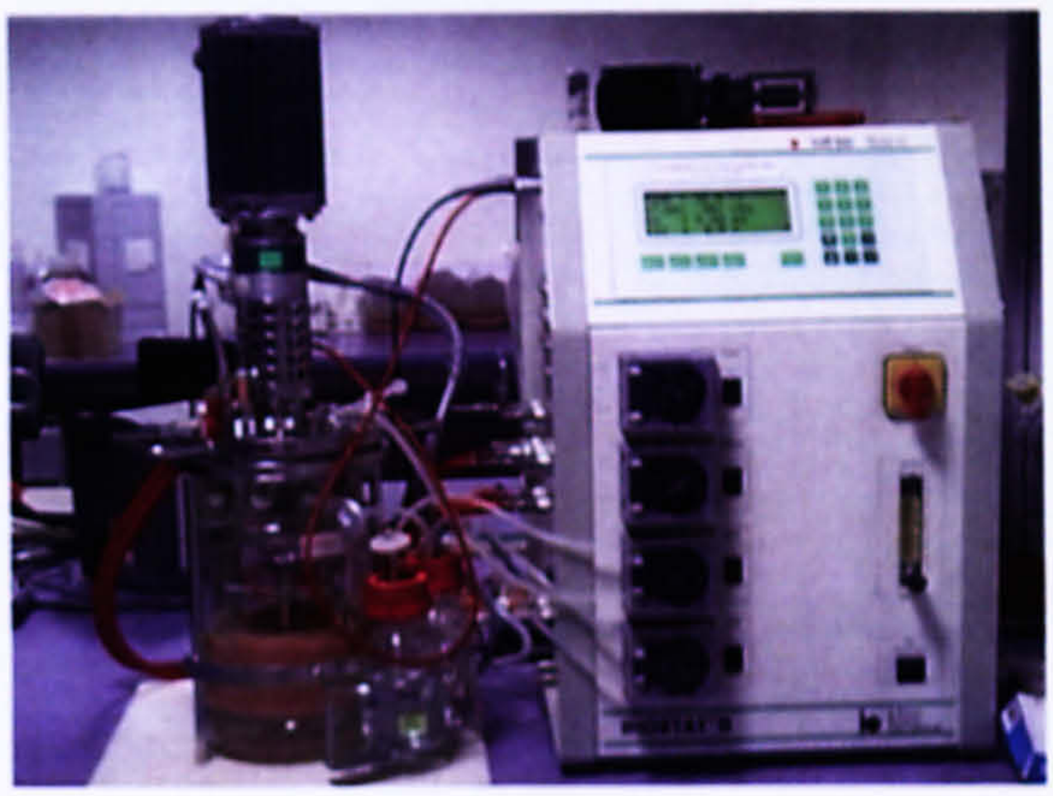

Figure 2: The 2L labscale benchtop fermentor with 1L working volume

3.2.4 Sampling

Samples of 20 ml were taken manually from fermentor vessel at 0 hours and 24 hours and stored at 4°C before further analysis. Three replicates were done.

3.3 Analytical Methods

3.3.1 Dry matter analysis

The dry matter analysis was done to determine its dry matter and its moisture content. The sago milk was shaked vigorously in a bottle for homogenous mixing. A dish with its cover was weighed. Sago milk of 100ml was poured into the dish with its cover and was reweighed. Three replicates were done and initial readings were recorded. Then, the samples were dried in the oven at 80°C for 24 hour. The samples were moved to desiccator to be cooled to room temperature and final reading of dish with cover and samples were recorded. This heating and cooling was done until the readings were constant.

$$\text{Dry matter} \quad \frac{(\text{Dried sample} + \text{Dish} + \text{cover}) \quad (\text{Dish} + \text{cover})}{\text{Volume of sample (ml)}} \times 100$$

$$\text{Moisture content} \quad \frac{(\text{Wet sample} + \text{Dish} + \text{cover}) - (\text{Dried sample} + \text{Dish} + \text{its cover})}{\text{Volume of sample (ml)}} \times 100$$

3.3.2 Reducing sugars analysis

This was based on DNS method (Miller, 1959). DNS reagent of 3 ml was added to 3ml aliquot of test solution. The mixture was heated for 15 minutes in boil water followed by addition of 1 ml of 40% of Rochelle salts to stabilize the color. The test tube was quickly cool under running water before being measure at 575 nm using a UV/Visible spectrophotometer. A standard curve was plotted using glucose as standards to read off glucose equivalent values. The conversion of starch into reducing sugar was expressed as DE which was the percentage of reducing sugars present on a dry solid basis.

$$DE\ (\%) = \frac{\text{Reducing sugar (g/L)}}{\text{Starch content (g)}} \times 100$$

3.3.3 Residual starch analysis

The residual starch analysis was determined by using iodine-starch colorimetric method (Nakamura, 1981). The iodine solution was added at 0.1ml to 1ml of appropriated diluted sample. Distilled water was added to the mixture to bring the volume to 10ml. Absorbance of the sample was measured at 590 nm by using a spectrophotometer (Ultrospec 1100 Pro). The residual starch analysis was calculated through a starch standard curve relating to the absorbance at 590nm (OD) to starch concentration (g/L).