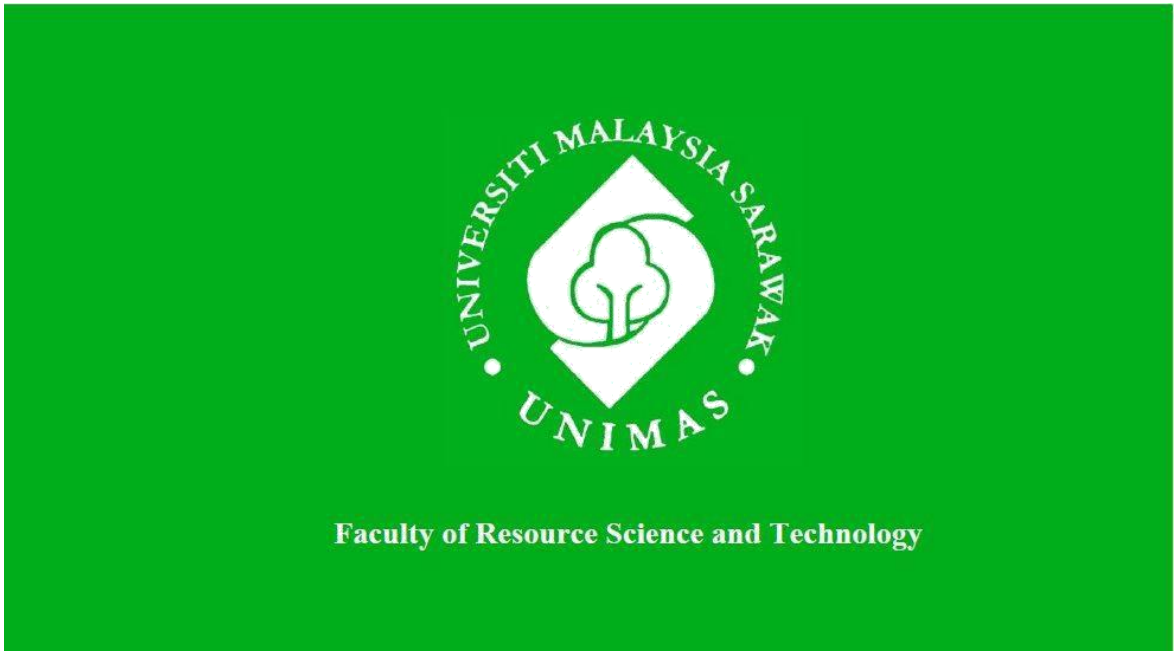

METAL IONIC COMPOUNDS SUPPLEMENTATION WITH SAGO HAMPAS HYDROLYSATE FOR LOW COST ETHANOL FERMENTATION MEDIA

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A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honour (Resource Biotechnology)

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DECLARATION

I declare that this thesis entitled "Metal Ionic Compound Supplementation with Sago Hampas Hydrolysate for a Low Cost Ethanol Fermentation Media", is my own work and all sources have been quoted and referred to have been acknowledge by means of complete references. It has been submitted and shall not be submitted to other university or institute of higher leraning.

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(Wilson Ngaring Anak Lim Bugau)

Date:

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LIST OF ABBREVIATIONS

S. cerevisae	*Saccharomyces cerevisae*
%	percentage
SHH	Sago ‘hampas’ hydrolysate
SmF	Submerged Fermentation
°C	Degree celcius
min	minute
rpm	Rotation per minutes
v/v	volume per volume
w/v	weight per volume
HPLC	High Performance Liquid Chromatography
OD	Optical Density
g/L	Gram per litre

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ABSTRACT

New era has arisen where we use the renewable resources to replace the demanding of fossil fuels as our main energy supplier. Ethanol fermentation also known as alcoholic fermentation helps in many areas such as agriculture, food, medical and chemistry. Through the process of fermentation, ethanol and carbon dioxide are produced. This study is aimed to produce the low cost ethanol from sago 'hampas' hydrolysate with the incorporation of metal ionic compound that was supplemented within the hydrolysate. Initially, 50 g/L of SHH were used for the fermentation media and the fermentation was conducted at room temperature at initial pH 5.5 to 5.6. Yeast or *Sacchromyces cerevisae* was used as microorganism and batch mode fermentation of 100 ml working volume was set up to run the experiment. Metal ionic compound that was used in this study are Mg^{2+}, Mn^{2+} and Zn^{2+}. The rate of fermentation were observe through the growth profile of the yeast, glucose consumption and finally the production of ethanol. As a result, zinc gives slightly higher ethanol production in using SHH as fermentation media with the help of *S. cerevisae*.

Key words: Sago 'hampas' hydrolysate (SHH), *Saccharomyces cerevisae*, ethanol, metal ionic compound,

ABSTRAK

Era baru telah bermula di mana kita menggunakan sumber yang boleh diperbaharui untuk menggantikan bahan api fosil sebagai pembekal tenaga utama. Fermentasi etanol juga dikenali sebagai fermentasi alkohol membantu dalam banyak bidang seperti pertanian, makanan, perubatan dan kimia. Melalui proses fermentasi, etanol dan karbon dioksida akan dihasilkan. Kajian ini, bertujuan untuk mengkaji penhasilan etanol kos rendah dari sagu hidrolisat 'hampas' dengan sebatian logam ion telah ditambah dalam hidrolisat itu. Pada mulanya 50 g /L SHH digunakan untuk media fermentasi dan fermentasi ini dijalankan pada suhu bilik, pH awal 5.5-5.6. Yis atau Sacchromyces cerevisia *telah digunakan sebagai mikroorganisma dan mod fermentasi 100 ml isipadu kerja digunakan untuk menjalankan eksperimen. Sebatian ion logam yang digunakan dalam kajian ini ialah* Mg^{2+}, Mn^{2+} *dan* Zn^{2+}. *Kadar fermentasi akan dilihat melalui profil pertumbuhan yis, penggunaan glukosa dan akhirnya pengeluaran etanol. Zink didapati menghasilkan etanol sedikit tinggi dengan menggunakan SHH sebagai media fermentasi dengan bantuan S. cerevisia.*

Kata kunci: Hidrolisat sago 'hampas' (SHH), *Saccharomyces cerevisia*, etanol, logam sebatian ion,

CHAPTER 1

INTRODUCTION

1.1 Background Study

Nowadays world starts to concern on the depletion of fossil fuel while struggling with the rapidly rising petroleum's price due to the higher demand of the mineral source worldwide. Fossil fuels are an important source of energy needed as supplying power for transportation as well as electricity. As an alternative, bioethanol seems to be the answer to replace the major dependent in fossil fuels. Ethanol is a renewable, locally manufactured alcohol fuel thru plant material, such as sago waste, sugar cane and banana waste (Energy efficientcy & renewable energy, 2010). Using ethanol can lessen greenhouse gas emissions. Plus, it is nonpolluting fuel that contain higher level of oxygen thus provide better combustion whilst decreasing the emission of two dangerous greenhouse gases known as carbon monoxide and hydrocarbon. Biofuels can either in gaseous or liquid form, bioethanol is in liquid form that made from the cellulose or hemi cellulosic biomass.

Ethanol is produced from the process known as fermentation. Through the fermentation process, glucose is converted to ethanol and carbon dioxide. *Saccharomyces cerevisiae* is usually used to convert the glucose to ethanol and carbon dioxide. Besides, it also produce lactic acid or acetic acid. Since *S. cerevisiae* does not possess amylase for the direct conversion of the starch to ethanol therefore the glucose must be obtained from the sago 'hampas' through a process known as hydrolysis. Fermentation does happen in the human's body that is when the muscle cell produces lactic acids which can result in the feeling of fatigue. In another perspective, ethanol fermentation seems very promising as another source

of energy as it can produce in a huge amount from raw materials or agricultural waste. Agriculture waste is cheaper and can be easily gained in a huge quantity.

Microelements play significant factors in cellular metabolism due to necessity as cofactors for a huge number of enzymes. Previous studies showed that metal ions are vital for all organisms and consequently ion transporters play a vital role in sustaining their homeostasis (Stehlik-Thomas, et al., 2004). Ammonium, magnesium and or zinc possess a defensive effect on growth, fermentation or cell viability which stimulates the degree of ethanol production (Nuangpeng, Laopaiboon, Srinophakun, Klanrit, & Laipaiboon, 2011). Those compounds help in producing the ethanol. As in this study, metal ionic compounds such as magnesium, zinc and manganese will be used to observe the effect of supplementation metal ionic towards ethanol production throughout the fermentation process. In this study sago 'hampas' will be used to obtain the glucose as it contains higher starch molecules. On its dry condition the sago contain 58% starch, which can provide glucose for ethanol production (Awg-Adeni, Abd-Aziz, Bujang, & Hassan, 2012).

1.2 Problem Statement

Which metal ionic compound (Zn^{2+}, Mg^{2+}, Mn^{2+}) in the media supplementation helps in boosting the ethanol concentration from the sago '*hampas*' hydrolysate (SHH) throughout the fermentation process at ambient temperature by *S. cerevisae*.

Hypotheses:

H_O: There is no significant difference of the supplementation metal ionic compound for the production of ethanol.

H_A: There is significant difference of the supplementation metal ionic compound for the production of ethanol.

1.3 Objectives

The objectives of this study are:-

1. To assess the effect of metal ionic (Zn^{2+}, Mg^{2+}, Mn^{2+}) supplementation in ethanol fermentation profile utilizing glucose in Sago 'hampas' hydrolysate.
2. To analyze the effect of metal ionic compound (Zn^{2+}, Mg^{2+}, Mn^{2+}) on the growth of *S. cerevisae*.

CHAPTER 2

LITERATURE REVIEW

2.1 Ethanol

Generally ethanol can be defined as an alcohol made by fermenting sugar and starch components of plant materials via yeast such as *S. cerevisae*. Nowadays, ethanol is used widely for transportation in its original form as an alternative to replace gasoline, however ethanol still mix with gasoline to improve transportation emissions (Hatfield, 1995). Produce ethanol said to be in a low cost because ethanol can be obtained from any agricultural waste (Awg-Adeni, Abd-Aziz, Bujang, & Hassan, 2012). Ethanol can be known as exotic synthetic oxygen containing organic chemicals because of its unique combination of properties as a solvent, germicides, antifreeze, fuels, depressants and chemical intermediate. The complete chemical formula for ethanol is CH_3CH_2OH. Ethanol consists of two components which is ethyl group (CH_3CH_2-) and alcohol group (-OH) (Fogler, 2005). This hydrocarbon burned completely to produce water molecules and carbon dioxide which is used directly for photosynthesis, resulting in zero net of carbon release and water molecules. According to Isaacs (1984), glucose can be hydrolyzed from cellulose with the aids of acid and enzymatically, however, there are few disadvantages to this method where it needs high temperature and at the same time low pH. This way requires expensive corrosive-resistant tools. In addition the conversion has limitation as the glucose degraded along the process.

2.2 Sago palm

According to Bujang (2001) the interesting features about sago palm is that it does not need any fertilizer to grow and the soil condition for it to grow mostly where the other crops cannot survive. In Sarawak approximately 90% is sago-planting areas where 75% of sago palm is located in Mukah. A matured sago palm is approximately 10-12 m high with 0.8-1

m diameter. It is planted using juvenile suckers and it grows more suckers just before it reaching its maturity state which is around 9-11 years (Awg-Adeni et al., 2012). Re-planting is not necessary since young palms will develop from other suckers thus ensuring a continuous supply of fresh logs in the future. Sago palm can be considered as a "survivor" as it needs minimal care to grow in in such a hard-to-survive swampy area.

2.2.1 Sago 'hampas'

Awg-Adeni et al., (2012) mentioned that sago 'hampas' is a starchy lignocellulosic by-product generated from pith of *Metroxylon sagu* or commonly known as sago palm. The quantity of sago 'hampas' produced from sago processing is depending on the quality of extraction. Sago 'hampas' approximately contain 66% starch and 14% fibre on dry condition. Sago 'hampas' will then undergo enzymatic hydrolysis through the process of liquefaction and saccharafication to obtain its glucose. The glucose is then used in the fermentation media as 'food' source (Bandaru, Somalanka, Mendu, Madicherla, & Chityala, 2006). For the percentage of composition content in sago 'hampas' please refer to **Appendix A**.

2.3 Submerged fermentation

Subramaniyam and Vimala (2012) had mentioned that submerged fermentation (SmF) consumes liquid substrates that flowing freely, for examples from molasses and broths. From the fermentation broth the bioactive compounds are secreted inside the broth. The substrates will consumed quickly thus it need to be replaced with nutrient. This technique of fermentation is favourable for microorganism such as yeast that need a high moisture content (Lin & Tanaka, 2005). Another advantage of this technique of fermentation is that the purification process of products is easier. SmF is usually used in the removal of secondary metabolites that essential to be used in liquid form.

2.4 High Performance Liquid Chromatography (HPLC)

Compared to column chromatography, High Performance Liquid Chromatography (HPLC) is far more improved chromatography analysis. Usually it is used to separate and detect any contaminants in samples. Via HPLC, any complicated or complex mixtures can be broken down into single or individual compound at their base level. Then it will be identified and quantified by the detectors and data handling. Another function of HPLC is to purify and quantify compounds that is in the samples (Kupiec, 2004)

2.5 Yeast

Yeast, also known as *Saccharomyces cerevisae* is one of the vital microorganism that aids in fermentation process as well as the production of ethanol. As any other living microorganism, yeast also prone towards it surrounding and can be affected by its environmental factors, gene type and also nutrition needed in order to sustain enough supply for the continuation of their life. According to Shafie (2009), a single cell of yeast is able to ferment glucose in its own weight about an hour time interval. The yeast will struggle if it's exposed too much in the factors mentioned earlier as it will affect their metabolism processes. According to Gibson (2011), yeast stress often result from its environment metabolic by-products such as ethanol and the naturally existing brew system in the yeast cell. Briggs, Boulton, Brookes, and Steven (2004), compromise that the rise in acidifying of yeast is affecting by transferring of nutrient rich medium such as magnesium. Stress circumstances which include quick temperature instabilities, high osmotic pressure, rises ethanol concentration, and nutrient starvation, adversely affect yeast cell dynamics; thus resulting in incompetent fermentation process. Although, recent study by Belloch, Orlic, Barrio, and Querol (2008), has revealed that low pH will lessen the stress of the yeast cell, nevertheless; it worsens the impact of other stress factors.

2.6 Metal Ionic Supplementation

Both nitrogen and carbon are vital in becoming nutrients for fermentation media. For yeast to develop, nitrogen is necessary. Besides, it is also an essential element that facilitates the production of ethanol and enables optimal acceptance of ethanol during the fermentation process. Yeast extract one of the composite nutrient widely used as a nitrogen basis need for yeast development as well as addition of nutrient for the manufacturing of ethanol and manufacturing of lactic acid. Other than both carbon and nitrogen there are other as equal as its important role towards the yeast development that are micro elements such as zinc (Zn^{2+}), magnesium (Mg^{2+}) and manganese (Mn^{2+}) (Saltukoglu & Slaughter, 1983). These micro elements also known as trace elements which are very crucial in promoting the growth of cell and for the fermentation of ethanol. Zn^{2+} for instance is essential for the growth of cell and also the metabolism of the yeast (Regalla & Lyons, 2003). Zhao, Xue, Ge, Yuan, and Bai (2009), stated that ethanol concentration and ethanol acceptance were significantly enhanced by Zn^{2+} supplemented culture. Mg^{2+} comprises in physiological role, development, metabolism and enzyme activity of yeast (Saltukoglu & Slaughter, 1983). It is a cofactor of some enzymes in yeast cells. Wang, Gao, Yang, and Xu (2007) reported that Mg^{2+} had a positive outcome on the production of ethanol. Mg^{2+} decrease the proton specifically the plasma membrane permeability by cooperating with membrane phospholipids causing the stability of the membrane bilayer. Hence, it improves on the ethanol acceptance of yeast. Furthermore, it gives positive feedback on the effect of ethanol efficiency in terms of time fermentation and the production of ethanol. On the other hand, Mn^{2+} is vitally necessary in the metabolism of *S. cerevisiae* that functions as one of the enzyme, known as pyruvate carboxylase, which is responsible for the formation of ethanol. The addition of Mn^{2+} can increase the growth of cell and also the concentration of ethanol (Loukin & Kung, 1995).

CHAPTER 3

METHODS AND MATERIALS

3.1 Materials

Sago ‘hampas’ (Hersden Sago Mill)

Dextrozyme (NOVOZYME, Denmark)

Stirrer (Stuart SS30, USA)

Saccharomyces cerevisiae (Mauripan Baking Industry, Australia)

Peptone (Qualikems, India)

Yeast extract (Becton, Dickinson and Company, USA)

KH_2PO_4 (monopotassium phosphate) (AnalaR NORMAPUR)

$MgSO_4.7H_2O$ (magnesium sulphate heptahydrate) (Hamburg)

$MnSO_4.H_2O$ (manganese sulfate hydrate) (Hamburg)

$ZnSO_4.7H_2O$ (zinc sulphate heptahydrate) (Hamburg)

Commercial glucose (AnalaR)

Shaker (EYELA, Rikakikai CO LTD, Japan)

250 ml Erlenmeyer flask (Schott DURAN, Germany)

High Pressure Liquid Chromatography system (Shimadzu, Kyoto, Japan)

Spectrophotometer (UV-1600, Shimadzu)

3.2 Methods

3.2.1 Preparation of Sago 'Hampas'

At 65 °C for 24 hours, the 'hampas' was left dried and grinded to the size where it can pass a 1 mm screen. According to Awg-Adeni et al., (2012) to test the moisture content of the 'hampas', dried samples will be taken, this is to make sure the amount of buffer that will be added for the process of enzymatic hydrolysis can be determined.

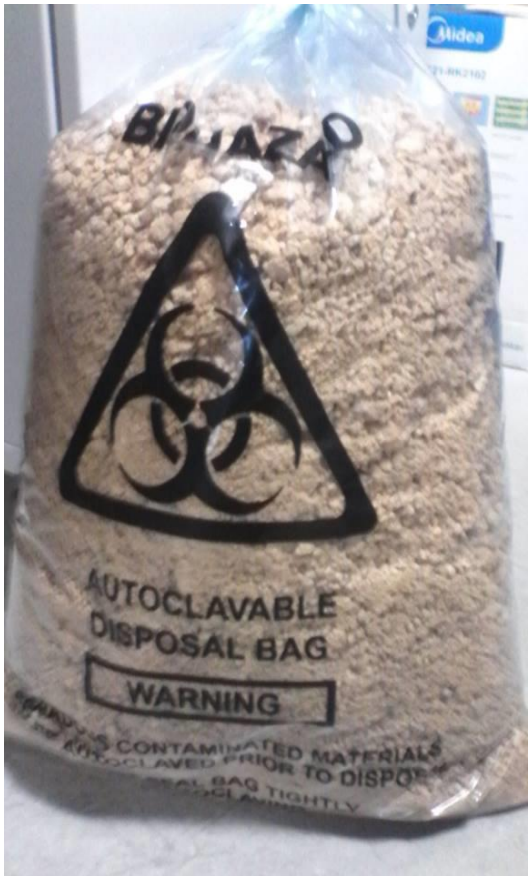

Figure 3.1 Sago 'hampas'

3.2.2 Saccharification of Starch in Sago 'Hampas'

In 0.1 M KH_2PO_4 buffer solution at pH 4, 7% (w/v) suspension of 'hampas' was put inside it. At 85-90 °C for 15 minutes the suspension was gelatinized. After that, add in liquozyme and further hydrolysis for 20 minutes. To ensure the mixture was mixed well, a stirrer was used. Then, it was left until it cooled down at 60 °C. Add in sprizyme for the saccharification

process. It then was left in the incubator shaker at 55 °C for 5 hours. Do the same thing for the next cycle for three cycle.

In every cycle of the hydrolysis, hydrolysate was gone through on a series of 100 mesh sieve filter, aimed to separate the hydrolysate from residual lignocellulosic fiber which then was centrifuged at 8000 rpm for about 10 minutes. As a result, the supernatant known as sago 'hampas' hydrolysate (SHH) was formed. This supernatant is taken off and further analyzed for reducing sugars and also the glucose content (Awg-Adeni et al., 2012).

3.2.3 Preparation of Inoculum and Microorganism

By using potato dextrose agar and yeast peptone glucose agar, the yeast or *S. cerevisae* is was cultured in both medium. Then, the yeast was transferred into a 100 mL inoculum media that contains 20 g/L glucose and 5g/L yeast extract. At 30 °C for 9 hours the inoculum is incubated and centrifuged at 5000 rpm for 10 minutes to obtain the cell pellets (Awg-Adeni et al., 2012).

3.2.4 Preparation of fermentation media

Inside the fermentation media, the contain were 5 g/L yeast extract, 2 g/L KH_2PO_4 (monopotassium phosphate), 1 g/L of peptone, 0.3 g/L $MgSO_4.7H_2O$ (magnesium sulphate heptahydrate), 0.3 g/L $MnSO_4.H_2O$ (manganese sulphate hydrate), 0.3 g/L $ZnSO_4.7H_2O$ (zinc sulphate heptahydrate), 1 g/L peptone and the concentration of SHH that was set at 50 g/L. Then the mixture was added to three different types of metal ionic compound which are magnesium, zinc and manganese. Four different conical flasks were set up with three of it were added with magnesium, manganese and zinc respectively. Another conical flask was set as a control where it was not added with any metal ionic compounds. Please refer to Appendix B, for the table that shows the fermentation media.

3.2.5 Conducting fermentation process

Batch fermentation was carried out at 30 °C for the process of fermenting. Along the process, the shacking rate was maintained at 100 rpm with the initial pH 5.5-5.6. With 100 mL working volume, the fermentation was took place in the 250mL of shake flask and incubated until the glucose residual is nearly zero. Samples were taken out and centrifuged at 10 000 rpm for 10 minutes at 4 °C to determine the glucose amount that have been consumed and the production of ethanol have been produced. Then, it is tested using HPLC. (Awg-Adeni et al., 2012).

3.2.6 Analytical method

3.2.6.1 Analysis of sago 'hampas'

At 105 °C the 'hampas' was left drying until a constant weight. The moisture contents of the sago 'hampas' then was tested.

3.2.6.2 Analysis of yeast

The dry weight of the cell was determined by centrifugation. 5 mL of sample was took and centrifuged at 5000 rpm at 10 minutes. The pellet form was washed using distilled water and left dry overnight at 105 °C. To get the final reading, the tube once again weighed (Lin et al., 2012). The formula to calculate the dry cell weight as follows:

$$\text{Dry cell weight, g/L} = \frac{(\text{wt. of dried centrifuge tube+cells,g}) - (\text{wt.of centrifuge tube,g}) \times 10^3}{\text{Sample volume (ml)}}$$

3.2.6.3 Analysis of glucose and ethanol

By using HPLC system (Shimadzu, Kyoto, Japan) with Shimadzu Liquid Chromatograph (LC-20AT) and Shimadzu Refractive Index Detector (RID-10A) the glucose amount that have been consumed and the ethanol production was tested. Aminex Fermentation

Monitoring Column 150 mm x 7.8 mm will be used and 5 mM H_2SO_4 (sulphuric acid) as a mobile phase with flow rate of 0.8 mL/min at 60 °C (Awg-Adeni et al., 2012).

CHAPTER 4

RESULTS AND DISCUSSION

This study shows how the effect of supplementation of metal ionic compound enhances the yield of ethanol production using *S. cerevisae* as microbe during the 24 hours fermentation period. Three metal ionic compounds were chosen which are magnesium, manganese and zinc. For the control of this study no metal ionic supplementation were added in the fermentation media. Metal ionic acted as the micro elements in the *S. cerevisae* development or growth. Thus it should enhance the production of the ethanol too. Before conducting the experiment, the glucose was obtained from the sago 'hampas' to be supplemented into the fermentation media. As the title of this study implies for a "low cost" production of the ethanol, thus commercial glucose was replaced with the glucose that we gained from the sago 'hampas' through the hydrolysis process. 7% of sago 'hampas' in dried basis was used in the hydrolysis process for three cycles. This fermentation was conducted in ambient temperature where the temperature was around 28-29 °C. The result was still promising even the temperature of the fermentation was below the yeast favorable condition. All the results from this experiment are presented as below.

4.1 Hydrolysis yield of Sago 'hampas' Hyrdrolysate

For every cycles of the hydrolysis, the efficiency conversion of the starch concentration was also increased. About 20% of water loss was occurred due to the evaporation and most of the water was suspended inside the substrate load. After the substrate was squeezed, the sago 'hampas' was tested for its moisture content. This is to calculate on how much water was loss during the squeezing process of the substrate that caused by the evaporation. The efficiency of the starch conversion was kept increasing in every cycle. It showed that 7% of sago 'hampas' was efficient enough to get enough glucose concentration for the fermentation