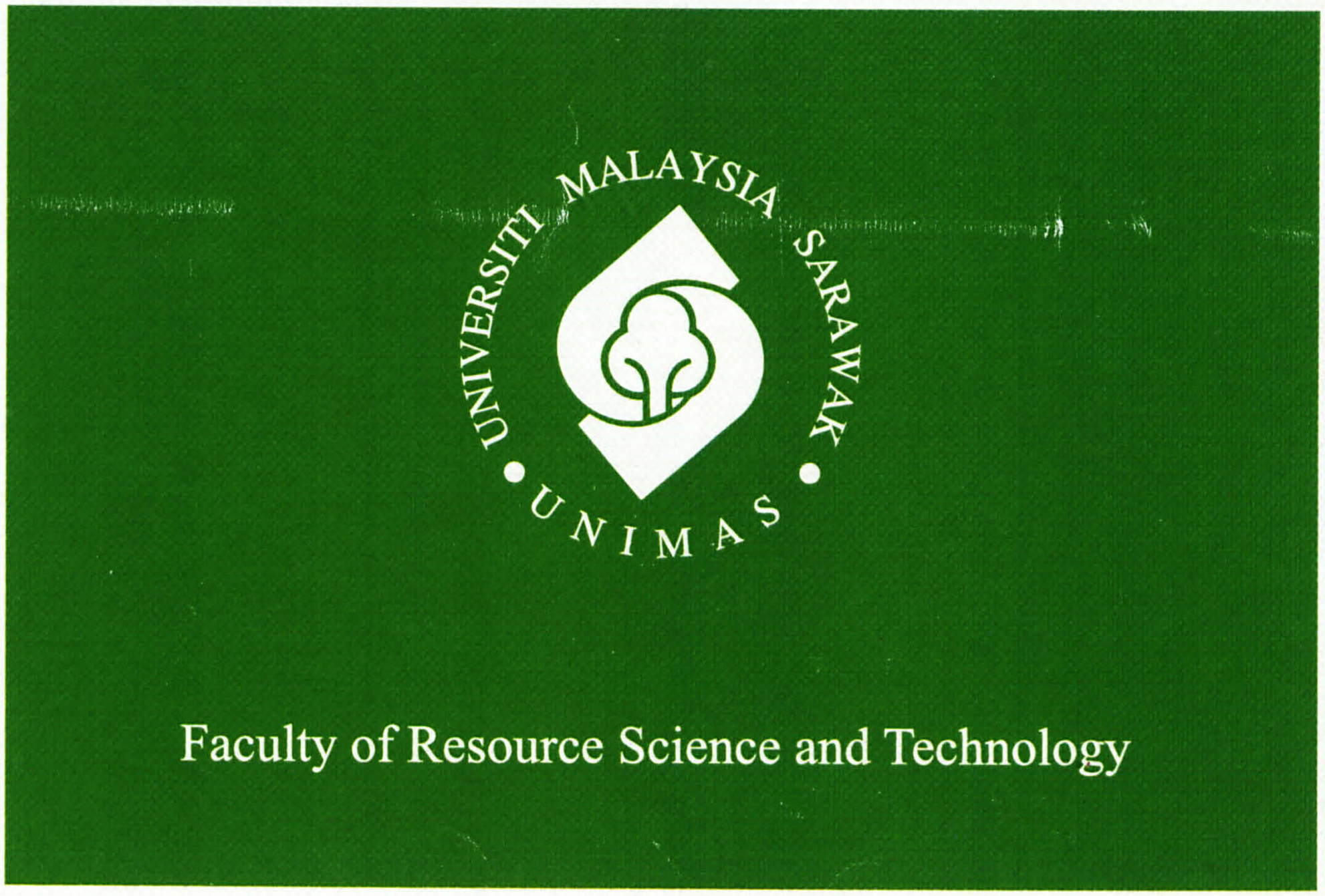

EFFECTS OF YEAST EXTRACT AND PEPTONE ON HYDROLYSED SAGO STARCH FOR BIOETHANOL PRODUCTION

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Effects of Yeast Extract and Peptone on Hydrolysed Sago Starch for Bioethanol Production

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

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DECLARATION

I hereby declare that this Final Year Project entitled "Effects of Yeast Extract and Peptone on Hydrolysed Sago Starch for Bioethanol Production" is based on my original work except for the quotations and citations which have been dully acknowledge also, declares that it has not been or concurrently submitted for any other degree at UNIMAS or other institution of higher learning.

Submission Date:

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LIST OF ABBREVIATIONS

Abbreviation	Description
%	Percentage
°C	Degree Celsius (temperature)
g/Kg	Gram per kilogram
g/L	Gram per liter
GC	Glucose Commercial
HG	High Gravity
HSS	Hydrolysed Sago Starch
mL	millilitre
NaOH	Sodium hydroxide
rpm	Rotation per minutes
S. cerevisiae	*Saccharomyces cerevisiae*
v/v	Volume (of solute) per volume (of solvent)
VHG	Very high gravity
w/v	Weight over volume

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Effects of Yeast Extract and Peptone on Hydrolysed Sago Starch for Bioethanol Production

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ABSTRACT

The subject of this study was the effect of yeast extract and peptone on production of glucose and ethanol productivity from hydrolysed sago starch (HSS) (100 g/L) using *Saccharomyces cerevisiae*. Since there are none nitrogen content was observed in sago starch, thus supplementation of nitrogen sources in the media are important. The method used was batch fermentation HSS with 100 mL initial volume with each was supplemented with nitrogen sources (yeast extract and peptone) and was maintained at room temperature (30 ℃ ± 0.5). The initial pH was set around 5.5-5.6 and fermentation was conducted in incubator shaker for 24 hours. Commercial glucose was used as control sample to compare it with HSS. In comparison of HSS with control sample, the gradual increase in dry cell weight of yeast can be observed in both fermentation media when supplemented with nitrogen sources. Based on the result from the experiment, yeast extract was proven to be the best nitrogen sources for ethanol fermentation due to high amount of ethanol produced (54.73 g/L) compared with peptone (14.27 g/L). Thus, the supplementation of yeast extract is important to achieve fermentation efficiency (99.24%) in fermentation of ethanol using HSS.

Key words: *Saccharomyces cerevisiae*, yeast extract, peptone, hydrolysed sago starch, ethanol

ASBTRAK

Subjek kajian ini adalah kesan ekstrak yis dan peptone terhadap penghasilan glukosa dan produktivi etanol daripada kanji sagu terhidrolisis (HSS) (100 g/L) menggunakan Saccharomyces cerevisiae. Oleh kerana tiada kandungan nitrogen diperhatikan di dalam kanji sagu, oleh itu penambahan sumber-sumber nitrogen di dalam media adalah penting. Kaedah yang digunakan ialah fermentasi kelompok daripada kanji sagu terhidrolisis dengan 100 g/L isipadu awal dengan setiap satu telah ditambah dengan sumber-sumber nitrogen (ekstrak yis dan peptone) dan kemudiannya dikekalkan pada suhu bilik (30 ℃ ± 0.5). pH awal telah ditetapkan sekitar 5.5-5.6, dan penapaian telah dijalankan dalam inkubator penggoncang selama 24 jam. Glukosa komersial telah digunakan sebagai sampel kawalan untuk dibandingkan dengan HSS. Apabila membandingkan HSS dengan sampel kawalan, peningkatan secara beransur-ansur dalam berat sel kering yis boleh diperhatikan dalam kedua-dua media penapaian sama ada ditambah dengan sumber nitrogen atau tidak. Berdasarkan hasil kajian, ekstrak yis telah terbukti sebagai sumber nitrogen terbaik untuk penapaian etanol kerana jumlah etanol yang dihasilkan tinggi (54.73 g/L) berbanding dengan peptone(14.27 g/L). Maka, penambahan ekstrak yis adalah penting untuk mencapai kecekapan penapaian (99.24%) dalam penapaian etanol menggunakan HSS.

Kata kunci: *Saccharomyces cerevisiae, ekstrak yis, peptone, kanji sagu terhidrolisis, etanol*

1.0 INTRODUCTION

Bioethanol are mainly produced from starch, sugar and lignocellulosic materials via fermentation technology (Zhang *et al.,* 2010). In order to optimize ethanol production from starchy materials a lot of research and new technology has been developed in recent years. Based on previous studies, it was proven that peptone and yeast extract are the best nitrogen sources, but due to the high cost it is not suitable to be used for large scale such as industrial manufacturing instead it is widely used for small scale only (laboratory scale) (Sridee *et al.,* 2011).

There are many types of yeast, however most of the yeast strain are not capable to directly ferment complex starchy materials (Lin & Tanaka, 2006). Therefore the most common one is the industrial strain yeast *S. cerevisiae*. This yeast can growth and produce ethanol if all the nutrients required in the media is enough and appropriate for the condition for the yeast to grow. Based on previous research, the ethanol productivity was enhanced more than 50% when nitrogen sources are added in the nutrient medium (Bafrncova *et al*., 1999). As stated in Pervez *et al*. (2014) study, *S. cerevisiae* was used to produce ethanol from glucose and after 24 and 48 hours of fermentation, and after 48 hours the result obtained were 84% of bioethanol. But in present studies, effects of carbon to nitrogen (C/N) ratio was investigated using recombinant *S. cerevisiae* YKU 131 (capable of expressing glucoamylase) to produce ethanol from sago starch. The results showed that 4.7 and 10.1 g/L ethanol was produced from 20 and 40 g/L sago starch respectively by using optimum C/N ratio which was 7.9 (Abd Aziz *et al.,* 2001).

As mentioned by Singhal *et al*. (2008), sago palm is also known as the starch crops of the 21st century' by most scientists. In Malaysia, Sarawak is known as world's biggest exporter of sago. Therefore, there are abundant of sago starch as source of ethanol

production. During the process, sago starch will undergo hydrolysis first where long polymer like chain of carbohydrate will be converted into glucose before fermentation process.

1.1 PROBLEM STATEMENT

There are many factors that can affect the yield of bioethanol production and one of it is the supplementation of nitrogen sources in fermentation media. None nitrogen content was observed in sago starch, therefore to ensure efficient bioethanol fermentation by yeast, nitrogen need to be supplemented in hydrolysate. Thus, research needed to be done on yeast extract and peptone. Thus, between these two, which one is more effective and when the highest ethanol will be produced in 100 g/L of HSS.

1.2 RESEARCH OBJECTIVES

The objective of this research is to study the effects of yeast extract and peptone in fermentation at 100 g/L concentration of hydrolysed sago starch (HSS) by *Saccharomyces cerevisiae* while using commercial glucose (CG) as control.

2.0 LITERATURE REVIEW

2.1 Nitrogen Sources

The best, low cost and most optimum amount of nitrogen sources is one of the improvement that are always being investigated in order to improve ethanol production. Nitrogen is necessary for yeast growth and multiplication, therefore the addition of nitrogen sources such as ammonium salts, urea and yeast extract in the media can increase final ethanol concentrations under VHG fermentation. Puligundla *et al.* (2011) stated that when excess assimilable nitrogen was added into batch VHG ethanolic fermentations, the final ethanol rise by 17% (to 103 g/L) achieved by using *S. cerevisiae*. In other reports, the ethanol productivity was enhanced more than 50% (fermentation time was halved to 28 h) with the addition of 12 g yeast extract, 20 g soya flour per litre, 3 g glycine and 0.3 g cell walls in the media (Bafrncova *et al.*, 1999).

Pereira *et al.* (2010) stated that with a medium developed based urea and corn steep liquor (CSL) for VHG ethanol fermentations enhanced final ethanol productivity and yeast viability (up to 330 g/L glucose) with a corresponding productivity of 2.4 $gl^{-1}h^{-1}$. It was proven that urea supplementation can balance the nitrogen deficiency and prolong the logarithmic phase of yeast cells thus resulting in high ethanol productivity during VHG fermentation (Theerarattananoon *et al.*, 2008).

With the addition of glycine it can stimulate yeast growth and the nitrogen inside it acts as osmoprotectant and supplement the high gravity media. Although proline and glycine betaine can also acts as osmoprotectants, but it was proven that glycine is more effective for high gravity fermentation (Thomas *et al.*, 1994).

However, the use of high concentration of substrate in the medium during the HG fermentation will cause the rise of osmotic pressure which will limit the yeast cells

efficiency (Zhang *et al.*, 2010). Research and studies have been conducted to find out the solution for this problem such as by adding yeast extract at 9 $g.L^{-1}$. But yeast extract is not appropriate to be used routinely because it is expensive. In another research, dried spent yeast (DSY) has been used in VHG ethanol fermentation from sweet sorghum juice due to its high mineral salts and nitrogen that contained in it. Based on the results obtained, the productivity and final ethanol concentration are higher when adding yeast extract into the VHG fermentation of ethanol (Zhang *et al.*, 2010). Table 1 below shows some nutrients and trace elements in yeast extract, peptone (HiMedia laboratory, India) and dried spent yeast (DSY) from Beerthip Brewery (1991) Co., Ltd., Bang Baan, Phra Nakhon Sri Ayutthaya, Thailand (Sridee *et al.*, 2011).

Table 1: Nutrients and trace elements in yeast extract, peptone and dried spent yeast (DSY)

Constituents (%)	Yeast Extract	Peptone	Dry Spent Yeast
Total N[a]	10.65	11.49	6.44
Total P[b]	1.15	0.80	1.25
Total K[c]	4.96	1.41	1.59
Total Na[c]	0.82	2.99	0.34
Total Ca[d]	0.024	0.013	0.067
Total Mg[d]	0.054	0.014	0.174
Total Fe[d]	0.0052	0.0102	0.0081
Total Mn[d]	ND*	ND*	ND*
Total Cu[d]	ND*	ND*	ND*
Total Zn[d]	0.0087	0.0040	0.004
Total Cl[e]	0.25	2.70	0.11
Total S[f]	0.35	1.00	0.35

a by Kjeldahl method; b Wet digestion (HNO3:HClO4) & Vanado molybdate method; c Wet digestion (HNO3:HCl O4) & Flame photometry method; d Wet digestion (HNO3:HCl O4) & Atomic absorption spectrophotometry method; e Dry ashing & AgNO3 titration method; f Wet digestion (HNO3:HClO4) & Turbidimetric method; ND*: not detectable.

*note: sources from: Sridee *et al*, 2011

2.2 Yeast

Yeast as unicellular eukaryotic microorganisms *S. cerevisiae* is an ethanol-fermenting organism. It is commonly known as baker's yeast and usually used in fermentation, in bakery products and beer industries where it converts fermentable sugars into ethanol and carbon dioxide. This yeast is used during fermentation to convert HSS into ethanol. Because of the availability of large amount of information about *S. cerevisiae* from development of recombinant DNA techniques, this yeast is widely used as a host cell for foreign gene products (Steyn & Pretorious, 1990).

Previous studies had focused on genetic modification of *S. cerevisiae* which allowed the direct ferment of starchy materials into ethanol in order to increase the production of ethanol. It involved genetic manipulation of *S. cerevisiae* for the expression of α-amylase and glucoamylase enzyme (Uchiyama *et al.,* 1995).

When there's enough nutrients provided in the medium, occasionally *S. cerevisiae* can ferment increased amount of sugars in the medium. But in present studies, effects of carbon to nitrogen (C/N) ratio was investigated using recombinant *S. cerevisiae* YKU 131 (capable of expressing glucoamylase) to produce ethanol from sago starch. The results showed that 4.7 and 10.1 g/L ethanol was produced from 20 and 40 g/L sago starch respectively by using optimum C/N ratio which was 7.9 (Abd Aziz *et al.,* 2001).

Many researchers studied on how to improve yeast viability by optimizing the media composition through adding supplement or by improving the fermentation process in order to enhance the yeast's ability to withstand stress during HG fermentation so that yeast able to produce high amount of ethanol. In order to decreased the fermentation time and enhance yeast viability, yeast growth, also the rate utilization of sugar, nitrogen sources are added into the fermentation media (Sridee *et al.,* 2011).

2.3 Sago Starch

Metroxylon sagu is commonly known as sago palms is the only example of commercial starch that derived from the stem of the palm while the other common starches are usually come from tubers, legumes, cereals and roots. There's a large amount of starch that could be found at the pith, or central portion of the stem, the sago palm's trunk. According to Ishizuka *et al.* (1995), the productivity of starch in sago palm was calculated to be 4 times of paddy. And compared to starch that derived from roots (cassava) the sago starch yield per unit area are about 17 times higher (Karim *et al.*, 2008).

According to (Chew *et al.*, 1999) for cultivation of sago, around 1.69 million hectares of peat swamp area in Sarawak are considered suitable for cultivation of this palm. Therefore there will be abundant amount of sago starch sources in Sarawak that can be utilized for bioethanol production. This starch has abundant uses, such as in food they are made into pearls which are then can be used in meals, in industries they are used in manufacturing soft drink, syrups and monosodium glutamate. Other alternative use of sago includes in manufacturing alcohol, citric acid, biodegradable plastics and ethanol (Chew *et al.*, 1999).

Currently, sago starch is used as substrate which is the main component in fermentation to produce ethanol. According to Awg-Adeni *et al.* (2012) ethanol is essential as a conventional non – renewable fuel replacement or fuel addictive and starch is one of the cheap substrate that can be used in ethanol production.

However in order to compete with oil palm industry, there are several things that needs to be enhance such as starch yield have to be increased, gestation period of sago palm need to be reduced and cost of production of sago also need to be reduced in order to match for oil palm in terms of profitability.

2.4 Hydrolysis of Sago Starch

In hydrolysis of sago starch process, long polymer like chain of carbohydrate will be converted into monomers of glucose. In this process, liquefaction and saccharification steps were carried out. Firstly, liquefaction steps involve the use of enzyme α – amylase, which will hydrolyses and gelatinized starch. The purpose of liquefaction process is to provide a partially hydrolysed starch suspension to retro gradation for further process which is known as saccharification (Ayier, 2005). Next, during saccharification steps, dextrines and oligosaccharides will be converted into glucose using glucoamylase enzyme. Glucose from sago starch will have to undergo fermentation process to convert it into ethanol.

2.5 Bioethanol

As the number of fuels consumers rises, the demand and consumption grows while the main source based on non – renewable fuels depleting over time. Other than that, a lot of other factors such as economic growth cause the oil price increasing. Present world also face with other problems such as global environmental concern that are worsening. Therefore, production of bioethanol from sugar, starch and cellulosic raw materials via fermentation are studied as alternative way to replace the non – renewable energy source.

According to Balat *et al.* (2013) it was proven that with an appropriate blending of conventional fuels with ethanol will helps in balancing the economic value of fuel price and also helps to promote a better environment. Compare to sugar rich substrate like sugar cane that provide readily fermented carbohydrate, starchy materials and cellulosic materials are more preferable to be used as substrate for the fermentation, because sugar cane is pricey, hard to obtain and is a seasonal crops (Abd-Aziz *et al.*, 2001).

Usually during the ethanol fermentation, the concentration of ethanol would increase when high amount of substrate loaded consequently enhance the efficient of downstream processing (Awg-Adeni *et al.*, 2012). However under high gravity fermentation, the high amount of starch loaded will affect the viability of yeast due to the osmotic pressure and ethanol toxicity.

It's an important parameter to be able to work at high-solid concentrations in the enzymatic hydrolysis process where it will affect bioethanol production in terms of economic viability and the balance of energy (Awg-Adeni *et al*, 2012). Therefore, during high gravity fermentation the yeast viability will be analysed at high substrate load (>100 g/L).

MATERIALS AND METHODS

3.1 Sago Starch

Sago starch was obtained from Herdsen Sago Industries Pusa, Sarawak in dry condition.

3.2 Hydrolytic Enzymes

For sago starch hydrolysis, Thermamyl-SC (thermostable α-amylase from *Bacillus licheniformis* 120 KNU/g) and Amyloglucosidase (AMG) purchased from Novozyme Biomass Kit (Denmark) were used throughout the experiments.

3.3 Preparation of Hydrolysed Sago Starch (HSS)

Initially, an amount of 100 g of sago starch powder was filtered and weighed and then suspended in 1 L of distilled water 10% (w/v). Thermamyl-SC (0.5 µL/g starch) was added into the starch slurry for liquefaction process. Next, starch slurry was incubated and stirred on hot plate (Figure 1) until it started to change colour (Figure 2) and when it started to change colour it was set to incubate at 80 - 90 °C for 2 hours. After that, liquefied suspension was left to cool down. Then, AMG (0.6 µL/g starch) was added to the liquefied suspension for saccharification and then it was incubated at 50 – 60 °C for 24 hours in Orbital Incubator Shaker GYROMAX™ 706 obtained from Hotech Instruments Corp. The hydrolysed sago starch after incubated for 24 hours is ready to be used in preparing fermentation media (Figure 3).

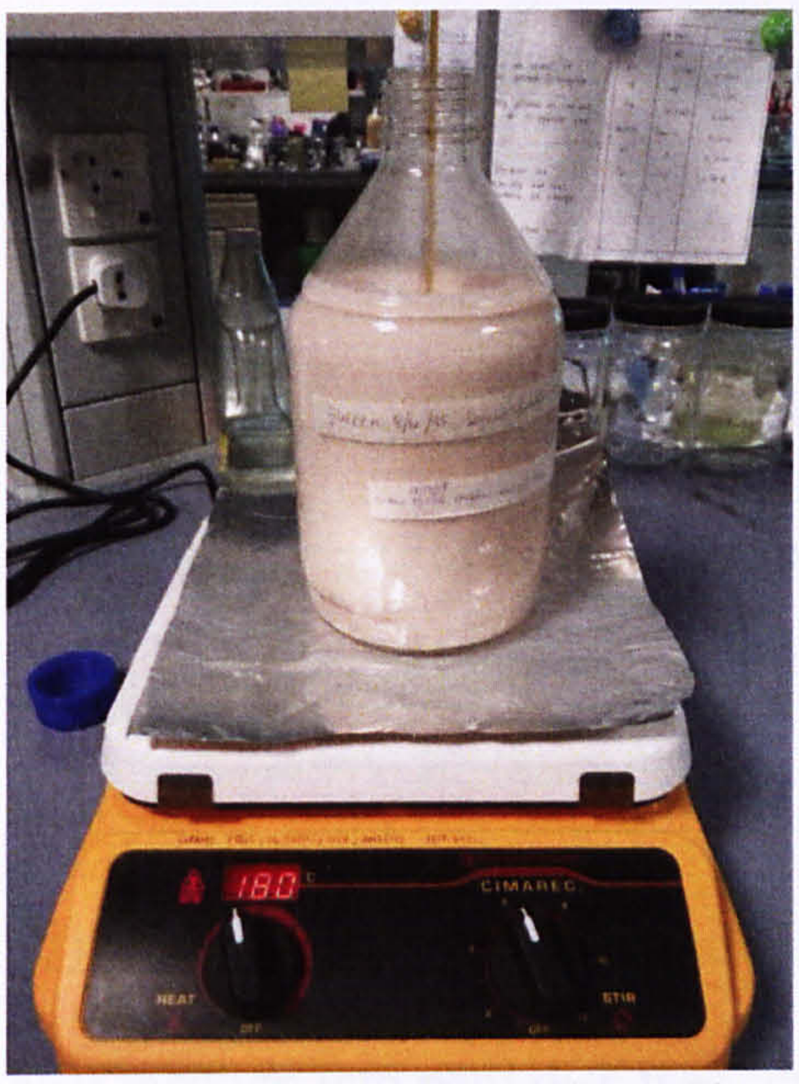

Figure 1: Hydrolysis of sago starch

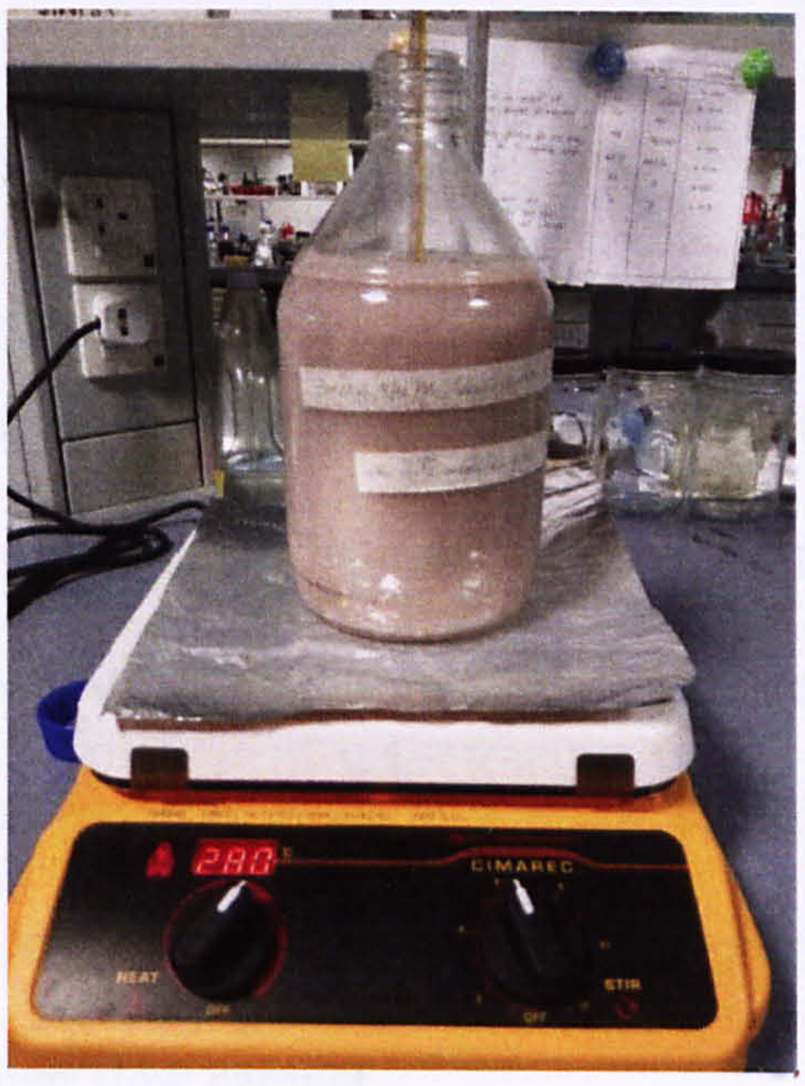

Figure 2: Starch slurry started to change colour

Figure 3: Hydrolysed Sago Starch (HSS) after incubated for 24 hours

3.4 Preparation of Microorganism and Inoculum

3.4.1 Potato Dextrose Agar

An amount of 500 ml of distilled water was heated on hot plate covered with aluminium foil. Then 39 g/L of Difco PDA powder was added to the hot distilled water and stirred using glass rod until it dissolved. Agar was cooked until the solution colour changed to transparent and PDA had fully dissolved. After that the agar medium was autoclaved at 121°C for 2 hours. The agar was left to cool down to 50 – 60 °C. The process of pouring agar medium into petri dish was conducted in a laminar flow cabinet.

3.4.2 Yeast Malt Broth Medium

An amount of 2.1 g yeast malt broth was added into 50 mL distilled water in a flask with two marbles included. This solution was autoclaved at 121 °C. Next, 1 g of a dry baker's yeast *S. cerevisiae* (Mauri-Pan brand) was added into the medium that was autoclaved after the temperature had decreased. Yeast malt broth medium was incubated in incubator shaker at 135 rpm for 24 hour.

One loop of yeast from the yeast malt broth medium was streak on PDA plates. The plates that were streaked were left in incubator at 30 °C for two days. From each plate a single colony was picked and streaks on new plates for sub-culture. Single colony (Figure 4) formed on the plates were picked as one loop and used for preparation of inoculum media.

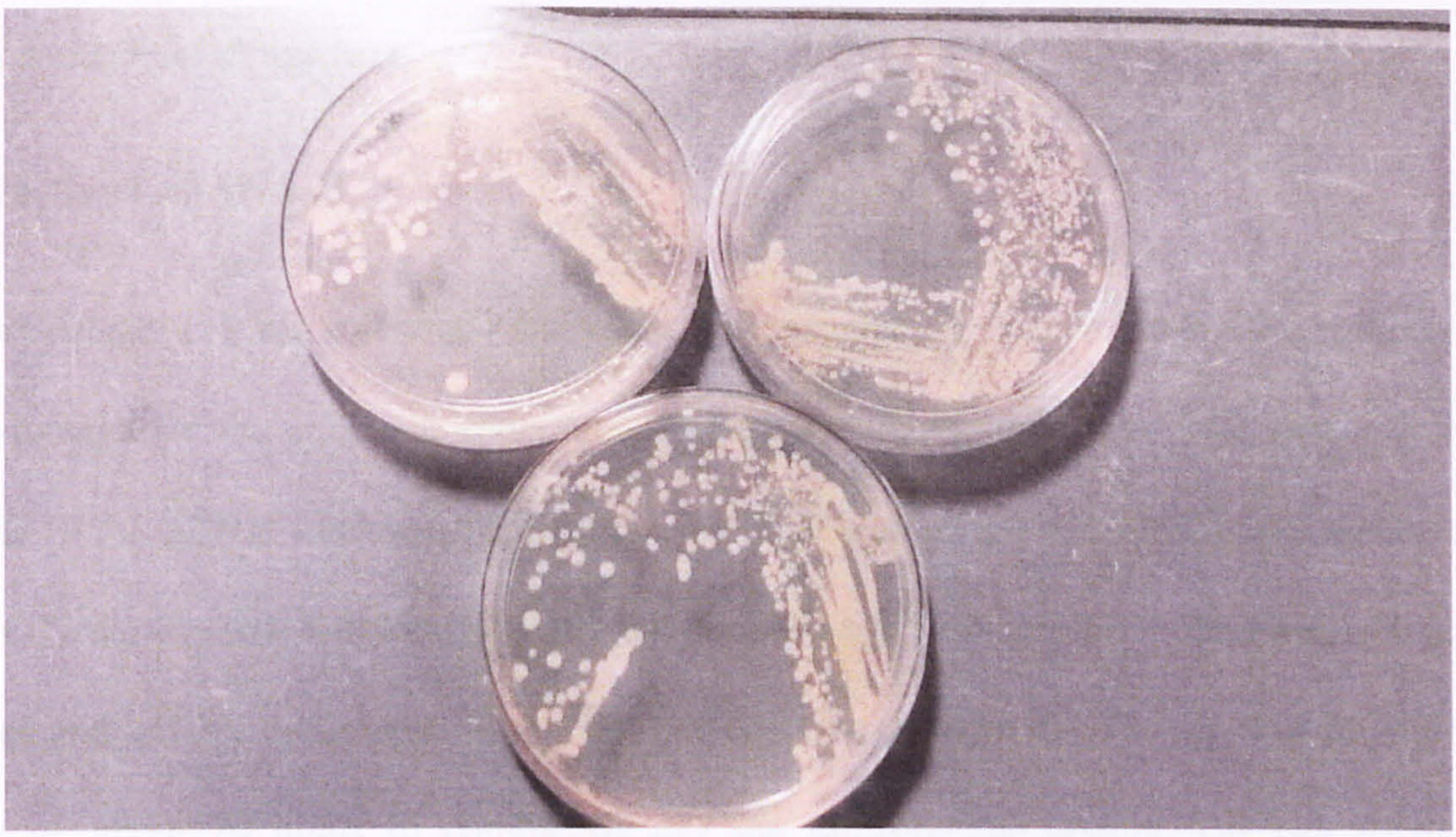

Figure 4: Single colony formed on plates

3.4.3 Preparation of Inoculum

One loop of yeast was selected from single colony and inoculated into inoculum media which contained 100 mL of 20 g/L of glucose and 5 g/L of yeast extract as carbon source. Then, this inoculum media was incubated in incubator shaker at 30 °C for 24 hours. After that, this inoculum was transferred into 50 mL centrifuge tube and centrifuged at 8000 rpm at 27 °C for 5 minutes. Supernatant was discarded while yeast pellet was transferred into fermentation media.

3.5 Preparation of Fermentation Media

Fermentation media was prepared 100 g/L from hydrolysed sago starch and supplemented with two types of nitrogen sources that are yeast exract 3.0 g/L and peptone 1.0 g/L accordingly. The pH for the media was set at 5.5 – 5.6.