Very High Gravity (VHG) Ethanol Fermentation from Sago Starch By

\textit{Saccharomyces cerevisiae}.

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Very High Gravity (VHG) Ethanol Fermentation from Sago Starch By
Saccharomyces cerevisiae.

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This project is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours (Resources Biotechnology)

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DECLARATION

I hereby declare that project is based on my original except for quotation and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at Universiti Malaysia Sarawak (UNIMAS) or other institutions.

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<td>%</td>
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</tr>
<tr>
<td>ºC</td>
<td>Celsius (temperature)</td>
</tr>
<tr>
<td>g/l</td>
<td>Gram per liter</td>
</tr>
<tr>
<td>g/Kg</td>
<td>Gram per Kilogram</td>
</tr>
<tr>
<td>v/v</td>
<td>volume (of solute) per volume (of solvent)</td>
</tr>
<tr>
<td>w/v</td>
<td>weight over volume</td>
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<tr>
<td>HSS</td>
<td>Hydrolysed SagoStarch</td>
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<td>VHG</td>
<td>Very High Gravity</td>
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<td>SSF</td>
<td>Simultaneous Saccharification and Fermentation (SSF)</td>
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*Saccharomyces cerevisiae*.

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ABSTRACT

Very high gravity (VHG) fermentation is a technology that required a fermentation of higher concentrations of substrate and, therefore, increased final ethanol concentration in the medium. The fermentation was using Hydrolysed Sago Starch (HSS) as a glucose sources and *Saccharomyces cerevisiae* as a microorganism to produce the ethanol. The effects of high concentration of glucose on ethanol concentration were investigated in this study. The sago starch of 100 g/l, 200 g/l and 300 g/l were used as substrate in this study, with the same concentration of glucose commercial were used as control. Fermentation was conducted at 30°C, pH 5.5 – 6.5 and agitation rate of 100 rpm which were control constantly throughout fermentation. The increase of glucose concentration had enhanced microbial growth in medium. The glucose consumption in all fermentation was not different significantly whereas the highest ethanol production, 145.06 g/l was achieved by using 300 g/l of HSS. This show that the ethanol production increase accordance with the utilization of higher HSS concentration. Therefore, the optimum HSS concentration for the highest ethanol production was 300 g/l in consideration of the highest ethanol production while consuming the same amount of electricity, time and labour as in fermentation by using 100 g/l and 200 g/l of HSS.

Key words: Very High Gravity, Hydrolysed Sago Starch, *Saccharomyces cerevisiae*, ethanol production.

ABSTRAK

Fermentasi VHG adalah satu teknologi di mana fermentasi menggunakan kepekatan substrat gula yang tinggi lalu meningkatkan konsentrasi akhir etanol di dalam media. Fermentasi ini menggunakan hydrolasat kanji sago sebagai sumber glukos dan *Saccharomyces cerevisiae* sebagai microorganisma untuk menghasilkan etanol. Kajian ini bertujuan untuk melihat kesan kepekatan glukosa yang tinggi terhadap konsentrasi etanol. Kanji sago yang berkepekatan 100g/l, 200g/l dan 300g/l digunakan dalam kajian ini dengan menggunakan glukosa komersial dengan kepekatan yang sama sebagai kawalan. Fermentasi yang dijalankan adalah dikawal sepanjang masa dengan menetapkan suhu operasi pada 30°C, pH 5.5-6.6 dan 100 rpm untuk kadar pendadukan. Peningkatan kepekatan glukosa di dapatkan dapat meningkatkan pertumbuhan mikrob. Penggunaan glukosa dalam semua fermentasi adalah tidak jauh bezanya manakala penghasilan etanol yang maksimum pula iaitu 145.06 g/l dicapai dalam fermentasi menggunakan 300 g/l HS. Ini menunjukan penghasilan etanol bertambah sejajar dengan penggunaan HSS yang lebih tinggi. Oleh itu, kepekatan HSS yang optimum untuk penghasilan etanol tertinggi kepekatanannya adalah 300 g/l HSS dengan mengambil kira penghasilan etanol yang berpekatan tertinggi di samping menggunakan kuantiti elektrik, masa dan tenaga pekerja yang sama seperti di dalam fermentasi menggunakan HSS yang berpekatan 100 g/l dan 200 g/l.

Kata kunci: gravity yang tinggi, kanji sago hydrolysat, *Saccharomyces cerevisiae*, penghasilan etanol.
CHAPTER 1

INTRODUCTION

In recent years, energy crisis is one of the serious cases in the world as the price of petroleum has risen with the decreasing of petroleum sources. The extreme usage of fossil fuels has lead to global warming due to the high emission of carbon dioxide and carbon monoxide from the transportation sector. Thus, the indigenous, affordable and clean source energy of biofuel has becomes an alternative source to replace the fossil fuel. Biofuels refer to the biomass-based fuels that used direct combustion in order to generate electricity production but the liquid biofuels is always be used in transportation sector. There are several type of biofuels likes bioethanol, biomethanol, vegetable oils, biodiesel, biogas, biosynthetic gas (bio-syngas), bio-oil, bio-char, Fischer–Tropsch liquids, and biohydrogen (Balat, 2011). Demirbas (2008) states the advantages of biofuels is that they are easily available from common biomass sources, it representing a Carbon dioxide cycle in combustion and have a considerable environmentally friendly potential. Besides, it benefits the environment, economy and consumers in using biofuels, and they are biodegradable and contribute to sustainability. Biofuel such as bioethanol is safe and not bring any harmful to living organisms because the complete combustion of bioethanol produces carbon dioxide and water. However, the production’s costs are still expensive for the wide use of ethanol as biofuel. Therefore, the development of fermentation processes using economical carbon source is important for the ethanol production in a commercial scale.
Very High Gravity (VHG) fermentation is a concept that has been further extended from the high gravity (HG) fermentation that was proposed in the 1980s (Pulligundla et al., 2011). VHG fermentation is a technology that provides great savings in process water and energy requirements through fermentation of higher concentrations of sugar substrate and, therefore, increased final ethanol concentration in the medium. However, in VHG technology the high sugar concentration affects the yeast metabolism that leads to decrease in final ethanol production. In practice, the increased ethanol concentrations at the end of fermentation can be realized by fermenting medium containing sugar in excess of 250 g/l in order to achieve more than 15% (v/v) ethanol compared with 10–12% (v/v), the range that is generally being observed in most distilleries all over the world (Pulligundla et al., 2011).

In Brazil, sugarcane is utilized for bioethanol production through fermentation process and distillation while in the United States and Europe mainly used starch from corn, and from wheat and barley, respectively. Bioethanol is produce from various substrates that is rich in fermentable carbohydrates such as corn, wheat, sugar cane juice and molasses, woody biomass, sweet and grain sorghum, barley, cassava, potato and sweet potato has been well-studied. Studies have shown that corn-based bioethanol yields 20–30% more energy, than is consumed in making it. On the other hand, sugarcane and cellulosic bioethanol yield renewable energy nine times worth the fossil energy used to produce them (Philippidis, 2008).
1.2 Objectives

The main objective of the study is to enhance ethanol concentration through VHG fermentation from hydrolysed sago starch from hydrolyses sago starch (HSS) by *Saccharomyces cerevisiae* while using glucose commercial (GC) as a control. Besides, to study the effects of high concentration of glucose on ethanol concentration, ethanol yields as well as ethanol volumetric productivity.
CHAPTER 2

LITERATURE REVIEW

2.1 Very High Gravity (VHG) Fermentation

In ethanol production, the medium containing glucose with more than 300 g/l of dissolved solid with other nutrients like free amino, nitrogen, yeast extract, sterols, etc. is used. Rapid fermentation and high final ethanol concentration are importance in ethanol industry (Puligandla et al., 2011). In VHG fermentation, the ethanol stress produced from the high concentration of substrate imposed on yeast can enhance the ethanol productivity. Wang et al. (1998) reported that, in VHG (dissolved solids > 300 g/l) wheat mash fermentation at 20°C for 200 h, maximum final ethanol concentration of 23.8 % (v/v) was obtained. An ethanol yield of 14.8 % (v/v) has been reported by Jones and Ingledew (1994a) from fermentation of blackstrap sugarcane molasses as sole substrate under VHG conditions (47.7 g/100 ml). While, studies on VHG technology of co fermentation of sweet stem sorghum juice and sorghum grain show that maximum final ethanol concentration was about 16.8 % (v/v) in 78 h (dissolved solid 340g/l) (Bvochora et al., 2000). According to Ingledew and Bayrock (2001) VHG was defined as the preparation and fermentation of mash containing 27 g or more of dissolved solids per 100 g of mash.
According to Laopaiboon et al. (2009) when the sucrose is used as carbon sources, the sweet sorghum juice containing total sugar of 280 g/l that give the maximum ethanol production efficiency with concentration, productivity and yield of 120.68 ± 0.54 g/l, 2.01± 0.01g/l/h and 0.51 ± 0.00 g/g respectively. When sugarcane molasses is used as carbon sources, the maximum ethanol concentration, productivity and yield show 109.34±0.78 g/l, 1.52 ±0.01 g/l/h and 0.45±0.01 g/g respectively. A list of starch substrates that have been tested and shown to be suitable for VHG fermentations is given in Table 1.
Table 1: VHG ethanol fermentation studies using different starch substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc. of dissolved solid</th>
<th>Maximum ethanol produced</th>
<th>Fermentation time (h)</th>
</tr>
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<tbody>
<tr>
<td>Wheat mash</td>
<td>35 % (w/v)</td>
<td>17.1 % (v/v)</td>
<td>72</td>
</tr>
<tr>
<td>Wheat mash</td>
<td>37.9% (w/v)</td>
<td>23.8% (v/v)</td>
<td>130</td>
</tr>
<tr>
<td>Hull –less barley</td>
<td>32% (w/v)</td>
<td>17.1% (v/v)</td>
<td>96</td>
</tr>
<tr>
<td>Oat</td>
<td>&gt;30% (w/v)</td>
<td>353.2±3.7 l/t (dry wt.)</td>
<td>72</td>
</tr>
<tr>
<td>Rye</td>
<td>32-34% (w/v)</td>
<td>434.5±5.1 l/t (dry wt.)</td>
<td>48</td>
</tr>
<tr>
<td>Rye and triticale</td>
<td>&gt;30 % (w/v)</td>
<td>15.7-16.1% (v/v)</td>
<td>96-120</td>
</tr>
<tr>
<td>Sweet &amp; grain sorghum</td>
<td>34% (w/v)</td>
<td>16.8% (v/v)</td>
<td>96</td>
</tr>
<tr>
<td>Corn mash</td>
<td>35% (w/v)</td>
<td>126-130 (g/kg)</td>
<td>72</td>
</tr>
<tr>
<td>Malto-dextrin</td>
<td>&gt;30% (w/v)</td>
<td>129 (g/l)</td>
<td>72</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>35% (w/v)</td>
<td>16.8% (v/v)</td>
<td>72</td>
</tr>
<tr>
<td>Corn mash</td>
<td>&gt;30% (w/v)</td>
<td>17% (v/v)</td>
<td>48</td>
</tr>
<tr>
<td>Barley (dehulled bold)</td>
<td>30% (w/v)</td>
<td>14.3% (v/v)</td>
<td>72</td>
</tr>
<tr>
<td>Potato mash</td>
<td>&gt;30% (w/v)</td>
<td>16.61% (v/v)</td>
<td>72</td>
</tr>
<tr>
<td>Finger millet mash</td>
<td>&gt;30% (w/v)</td>
<td>15.6% (v/v)</td>
<td>72</td>
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<tr>
<td>Cassava starch</td>
<td>40% (w/v)</td>
<td>15.03% (v/v)</td>
<td>72</td>
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<tr>
<td>Sweet potato mash</td>
<td>&gt;30% (w/v)</td>
<td>~ 17.0% (v/v)</td>
<td>36</td>
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</tbody>
</table>

*note: sources from: Puligundla et al., 2011*
2.2 Yeast

*Saccharomyces cerevisiae* is universal organism in ethanol production by using starch and sugar substrate. Besides, *S. cerevisiae* be able to produce ethanol as high as 18 % of the fermentation broth. This yeast also can grow on simple sugars like glucose and on the disaccharide sucrose. Research has revealed that many strains of *S. cerevisiae* can tolerate far higher concentrations of ethanol much better than previously believed, without any conditioning or genetic modifications (Casey and Ingledew, 1986). The metabolism by this organism produces sugars such as glucose, fructose, mannose, galactose, sucrose, maltose and maltotriose. Apart from that, *S. cerevisiae* is inhibited by its own product, ethanol. *S. cerevisiae* can grow better at low pH to avoid contamination with other bacteria but not be able to utilize xylose for growth or fermentation. *S. cerevisiae* in VHG fermentation (280 g/l and 320 g/l glucose) shows the maximum ethanol concentration at 104.68 g/l as well as high ethanol productivities rather than normal gravity fermentation (Laopaiboon *et al.*, 2009). Based on research done by Watanabe *et al.* (2010) NFRI3225 strain of *S. cerevisiae* produced ethanol from potato mash at the fastest rate and in the highest volume, 13.7% (w/v) among the tested strains. While, the maximum productivity and ethanol yields were 9.1 g/l/h and 92.3%, respectively. Production of ethanol under very high-gravity cassava mash by *S. cerevisiae* during simultaneous saccharification and fermentation (SSF) show an increased of final ethanol concentration from 8.21 % (wt. %) to 15.03 % (wt.%) (Yingling *et al.*, 2010).
2.3 Sago starch

Sago palm is an important economic species and is now grown commercially in Malaysia, Indonesia, Philippines, and New Guinea for the production of sago starch (Saifuddin and Hussain, 2011). Among the starch from various origins, the use of sago starch is more economically attractive due to the abundance of sago palm in Sarawak. Therefore, sago starch is used in this study.

The sago starch is obtained from the stem of palm and according to Ishizuka et al. (1995) the trunk of sago palm contain large amount of starch and four times more than paddy rice. *Metroxylon sagu* and *Metroxylon rumphii* is the type of sago palm that usually used in industrial sago starch, for manufacture of glucose, dextrines, monosodium glutamate and industrial alcohol and for further conversion of starch. Sago starch represents an alternative cheap carbon source for fermentation processes that is attractive out of both economic and geographical considerations (Abd-Aziz, 2002). Fermentation of ethanol use a sugar-rich substrate as it is in fermentable form such as corn and sugarcane. However, corn and sugarcane is more expensive (Abd-Aziz et al., 2001) than sago starch. As pointed out by Jong (2002), sago starch can compete with other starch produced by annual crops as sago palms have century-long economic life span. In a study on simultaneous saccharification and fermentation (SSF) of ethanol from sago starch with co immobilized amyloglucosidase (AMG) (immobilized on powdered chitin) and *Zymomonas mobilis* MTCC 92 by submerged fermentation, a maximum ethanol concentration of 55.3 g/l was obtained using a starch concentration of 150 g/l (Bandaru et al., 2006).
2.4 Bioethanol

Bioethanol have been used as fuel for transport in Germany and France as early as 1894 while Brazil has utilized bioethanol as a fuel since 1925. The cost to produce bioethanol are become more expensive than petroleum fuel but after World War II, (Demirbas et al., 2009). Bioethanol is a liquid biofuel derived from renewable sources on different biomass feedstock and conversion technologies. Nowadays, ethanol is produced from renewable and cheap agricultural products that have a function to reduce the greenhouse effect to eliminate smog from the environment. Sugar or starch crop and lignocellulosic biomass is the sources for bioethanol production. Sugar cane is the main feedstock for bioethanol production in Brazil, while corn and sugar beet are the major resources in US and Europe respectively. Production of bioethanol by traditional technologies is included in „1st Generation Biofuel”. Reddy and Reddy (2005) found that production of ethanol by the finger millet flour in high gravity sugar (30–40%, w/v) not only reduced the fermentation time (from 5 to 3 days) but also enhanced the ethanol concentration from 10 to 15% (v/v) by better utilization of sugar. Apart from that, it was found that palmyra jaggery (sugar syrup from the palmyra palm) is a suitable substrate for the production of high concentrations of ethanol using S. cerevisiae NCIM 3090 by submerged fermentation. A maximum ethanol concentration of 129.4 g/l was obtained after optimizing media components and conditions of fermentation (Ratnam et al., 2005).
CHAPTER 3

MATERIAL AND METHODS

3.1 Sago starch

Industrial grade sago starch for use in hydrolysis as substrate in the fermentation process will be obtained from local market.

3.2 Preparation of hydrolysed sago starch (HSS)

Sago starch in the samples was hydrolyzed prior to residual glucose analysis, sago starch was hydrolyzed according to the method reported by Bujang et al., (1999). Two enzymes, Termamyl-120L (thermostable 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) will be used for hydrolyzing process. A two-step enzymatic hydrolysis, liquefaction and saccharification processes is carry out. The first step is liquefaction, which is conversion of a concentrated suspension of starch granules into a solution of soluble dextrins of low viscosity for convenient handling in ordinary equipment and for easy conversion to glucose by dextrozyme.
Typically, a suspension of starch in water is adjusted to pH 6 - 6.5 by addition of 1 M NaOH, optimal for α amylase. The suspension is heat up to 90 – 100°C, where the starch is immediately gelatinized. After that, 0.5 µl Termamyl-120 L per gram of starch add with 30 gm calcium ion ( per 1 kg of starch) that serve as an activator and stabilizer. The starch slurry is maintained at the temperature of 80 – 90 °C, for 1-3 hour. The objective of second step, saccharification is to achieve maximum conversion of dextrin to D-glucose. Here, the pH of the suspension was adjusted to 4 - 4.5, and lowering the temperature to 60°C. The 0.6 µl Dextrozyme (per gram starch) is add to the slurry. The conversion yield of starch into reducing sugar by enzymes will be calculated based on glucose concentration per starch concentration. The HSS will be used on glucose sources for VHG bioethanol fermentation.
3.3 Microorganism and inoculums preparation

3.3.1 Agar preparation

3.3.1.1 Potato Dextrose Agar (PDA)

For preparation of PDA agar plates, 3.9 g of Difco™ PDA powder was dissolved in 100 ml of distilled water. Magnetic stirrer was used to stir the agar medium at high temperature. When the turbid solution changed to transparent, this mean the agar had fully dissolved. Then the agar medium was autoclaved at 121°C for 20 minutes. The agar medium was let to cooled down to about 50 - 60°C and then poured into petri dish. This was done aseptically in an ERLA CFM Series laminar air flow cabinet.

3.3.1.2 Yeast Extract, Peptone and Glucose (YPG) Agar

For preparation of YPG agar plates, 5 g of Difco™ yeast extract, 10 g Bacto™ peptone, 10 g of glucose and 9 g of agar were dissolved in 500 ml distilled water. Magnetic stirrer was used to stir the agar medium at high temperature. When the turbid agar medium changed to transparent, this mean the agar had fully dissolved. The agar medium was then autoclaved at 121°C for 20 minutes. The agar medium was let to cooled down to about 50 – 60 °C and then poured into Petri dish. This was done aseptically in an ERLA CFM Series laminar air flow cabinet.
3.3.2 Inoculum preparation

Several colonies of Yeast from YPG agar plates were transferred into inoculums media. The preparation media for inoculums are 20 g/l commercial glucose and 5 g/l of yeast extract with working volume of 100 ml. The pH for inoculums was set at 5.5. The prepared inoculums media was autoclaved for 45 minutes at 121 °C in an effort to prevent any microorganisms other than yeast from growing. The inoculum was incubated at 30 °C for 15 hour before being centrifuge. The centrifugation (8000 rpm, 5 min) was performed to obtain the pellet of yeast. The yeast was transferred into fermentation media.

3.4 Media for fermentation

Media for fermentation contained (g/l); yeast extract 3; peptone 1; (NH₄)₂SO₄, 1.4 ; KH₂PO₄ 2; CaCl₂, 0.3; MgSO₄.7H₂O,0.3. Three different glucose concentrations (100 g/l, 200 g/l and 300 g/l) was used in fermentation media with working volume of 100 ml. The pH for media will be 5.5-5.6 and temperature at 30 °C whereas agitation at 100 rpm.