Ethanol Fermentation Using Glucose Derived from Sago Starch by *Saccharomyces cerevisiae* under Very High Gravity Condition (VHG)

Muhamad Supri bin Ajim  
(24173)

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Muhamad Supri B. Ajim
(24173)

Programme: Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

In ethanol fermentation the use of microorganisms which can stand stressing conditions such as very high gravity (VHG) to produce high concentration of ethanol is desired. Therefore, a study was carried out to investigate the performance of *Saccharomyces cerevisiae* SCI-1 to produce ethanol in batch fermentation under VHG conditions. To improve the productivity, the effect of the soya flour supplementation on the yeast growth as well was studied. The fermentations were carried out at 30°C, pH 5.5±0.5, in 1-L fermentor under an agitation rate of 400 rpm. VHG condition was assessed using 300.6g/l for unsupplemented and 296.52g/l for supplemented of hydrolyzed sago starch. The results showed that, the yeast has an excellent performance to ferment glucose under VHG conditions. The production of ethanol was no significant different at p<0.05 when soya flour was added to the media even though ethanol increase production from 109.616g/l to 121.201g/l when compared to the fermentation without addition of soya flour.

Keyword: *Saccharomyces cerevisiae* SCI-1, Hydrolyze Sago Starch (HSS), Very High Gravity, soya flour.

ABSTRAK

Dalam proses penapaian etanol penggunaan mikroorganisma yang boleh bertahan dalam keadaan tekanan seperti gravity yang sangat tinggi (VHG) untuk menghasilkan kepekatan etanol yang tinggi telah dikehendaki. Oleh itu, satu kajian telah dijalankan untuk menyiasat prestasi *Saccharomyces cerevisiae* SCI -1 untuk menghasilkan etanol dalam penapaian di bawah keadaan VHG. Untuk meningkatkan produktiviti, kesan tambahan tepung soya kepada pertumbuhan yis juga telah dikaji. Penapaian telah dijalankan pada suhu30±3°C, pH 5.5±0.5, fermentor 1-L di bawah kadar pengadukan 400 rpm. Keadaan VHG telah dinilai menggunakan 300.6g/l medium tanpa soya dan 296.52g/l medium ditambah soya menggunakan kanji sagu terhidrolisis. Hasil kajian menunjukkan bahawa, yis mempunyai prestasi yang sangat baik untuk menapai glukosa di bawah keadaan VHG. Pengeluaran etanol adalah perbezaan yang tidak bererti pada p<0.05 apabila tepung soya telah ditambah ke dalam media walaupun peningkatan pengeluaran etanol dari 109.616g/l kepada 121.201g/l apabila dibandingkan dengan penapaian tanpa tambahan tepung soya.

Kata kunci: *Saccharomyces cerevisiae* SCI-1, Kanji Sagu Terhidrolisis (HSS), Graviti Sangat Tinggi, tepung soya.
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1.0 INTRODUCTION

Alcoholic fermentation is the anaerobic process where it involved the conversion of sugar into ethanol and carbon dioxide as well release heat. Ethanol can be produced by fermentation process in which raw materials such as sugars, starches and cellulose can be used as substrates.

\[
\text{Glucose} \rightarrow \text{Ethanol} + \text{Carbon Dioxide} + \text{Heat}
\]

Countries such as USA, Brazil, and Thailand used corn grains, sugarcane, tapioca and sugarcane molasses respectively as their source of raw materials (Goksungur and Zorlu, 2001, Laopaiboonet al., 2007, Schaffert, 1995). United States lead the world in global ethanol production and approximately that 30% of the US total corn crops used to produce ethanol where they use wet and dry milling method. Meanwhile Brazil come second largest global ethanol production, and first sustainable biofuels economy and industry leader in biofuels. Brazil's have the sugarcane ethanol program whereby it is the most successful alternative fuel program to date and become the model for other countries (Soybean and corn Advisor, Inc 2009).

High degree of efficiency of fuel alcohol can be achieved from fermentation of starch and other carbohydrate-base materials and the ethanol production can yields up to 90 to 95% of the theoretical maximum have been study under industrial condition (Thomas and Ingledew, 1990).

Usually, the fermentation of ethanol is carried out under normal gravity (NG) condition (Thomas et al., 1996) where the process under this condition was time
consuming, high cost, and use high energy. According to Bayrock and Ingledew (2001), very high gravity (VHG) technology has been studied and proved to increases the productivity and cost effectiveness of ethanol production. VHG is the condition where the preparation and fermentation to completion of mashes containing 27g or more dissolved solid per 100g mash (Pradeep, 2010). Improvement that can be achieved by using VHG technology is that it can reduce the fermentation time as well as increasing the rate of production and increasing the concentration of alcohol (Thomas and Ingledew, 1990). Many researches has been conducted based on the VHG condition and using many approaches from different aspect such as glucose derived from, species of bacteria and fungi, media supplementation, pH range and as well as temperature range.

However, fermentation under VHG condition has few limitations. When concentration of ethanol or wort increases, the yeast will be vulnerable due to the increasing of osmotic pressure and toxicity environment of ethanol production (Pátková et al., 2000; Erten et al., 2007). After certain of period of time where the yeast have utilized high concentration of glucose under high gravity condition, the production and concentration of ethanol increase as well and thus create the environment of high osmotic pressure and toxicity to the yeast itself. Hence the environmental stress will cause a stuck or sluggish fermentation (Thomas et al., 1996; Bafrncova et al., 1999) and decrease in viability of the yeast (Almeida et al., 2001). Due to that the yeast unable to further the fermentation process even though there are still more glucose is available. Substrate inhibition may be occurred under the condition (Thomas et al., 1996). Preparation and fermentation of substrate containing 27g and beyond that dissolved solid per 100 ml consider as high gravity (Pradeep, 2010).
In this study a hydrolyzed sago starch (HSS) were used as a raw material and *Saccharomyces cerevisiae* CSI-I type strain metabolized the glucoses and convert it to produce ethanol under VHG condition in batch fermentation. The aim of this study is to investigate the rate of ethanol fermentation under VHG condition using strain of *Saccharomyces cerevisiae* CSI-1 using batch fermentation technique.

**Objectives of the study**

1. To study the performance of *Saccharomyces cerevisiae* CSI-1 in term of ethanol production under VHG condition.
2. To determine the effect of soya flour as enhancer of the cell growth and ethanol production.

**The hypothesis of the objectives studies were:**

**Ho:** *Saccharomyces cerevisiae* CSI-1 is able to stand VHG conditions to growth and produce high ethanol concentration without the supplement of soya flour as growth enhancer.

**Ha:** *Saccharomyces cerevisiae* CSI-1 is able to stand VHG conditions to growth and produce high ethanol concentration with the supplement of soya flour as growth enhancer.
2.0 LITERATURE REVIEW

2.1 Very High Gravity Fermentation

VHG, at present, emerge as a promising fermentation technology to cut down the increasing energy cost by allowing the significant increasing of the final concentration of ethanol from 7-10% up to 15-18% (v/v) or even more (Pradeep et al., 2009) and it is a new perspective technology for the ethanol production (Bafrcncova et al., 1999). VHG was defined as the preparation and fermentation of mashes containing higher than 270g of dissolved solids per litre (Bayrock and Ingledew, 2001). Fermentation of high sugar mashes has been considered impractical in industries of alcohol because of yeast viability and incomplete or stuck fermentation (James and Ingledew, 1993). Many researches has been conducted on VHG for fermentation of ethanol regardless the composition nutrient in medium, raw materials employed, microorganism used, temperature ranges, and addition of some additives; all work of study aimed to increase and produce as high ethanol concentration can be and at the same time reduced the time rate and cost of production. The main ideas that have been issued in many researches in VHG fermentation are the toxicity of yeast toward the high ethanol concentration, the stuck and incomplete fermentation and the high energy, time and cost consumption.

In North America and Europe, starchy-rich plant such as wheat, corn, barley and oats were used as predominant source of fuel ethanol and research study works (Pradeep et al., 2009). It has been report that, a successful VHG fermentation was tested on oats, barley, rye and triticale (Wang et al., 1999b). Abd-Aziz (2002) suggested the utilization of sago palm for ethanol production in the case of Malaysia.
James and Ingledew (1993) worked on wheat mashes to study the alcohol production for the optimization of temperature and found that as the temperature increased from 17°C to 33°C, the time requires to end the fermentation decreased, but increased as the concentration glucose was raised from 14.0 to 36.5g/100 ml. Their studies conclude that optimum temperature for maximum ethanol production was 27°C for wheat mashes, fermented by with supplement of urea for efficient VHGA fermentation. In addition assimilable nitrogen such Free Amino Nitrogen (FAN) also being highlight as an important component in fermentation media as it influence the ethanol tolerance of yeast and rate of ethanol production because it supplied the cell to make the structural protein and enzyme to enable optimum metabolisms and so increase the cell mass accumulation (Bafrncovaet al., 1999).

2.2 Sago palm

According to Heywood (1993), sago palm belong to Arecales Nakai orders, family of *Palmae* Jussieu, subfamily of *Calamoideae* Griffith, subtribe of *Metroxylinae* Blume and genus of *Metroxylon* Rottboell (Uhl and Dransfield, 1987). Among tropical crops, sago palm is one of the few that have the ability to thrive and live in the swampy peat environment (Johnson, 1977; Ruddle, 1977). Local farmer in Sarawak classifying sago palm into few stages of physiological growth that is Plawei (palm at maximum vegetative growth), Plawei Manit (inflorescence emerging), Bubul (inflorescence developing), Angau Muda (flowering), and Angau Tua (fruiting) (Lim, 1991). In Pei-Lang et al. (2005) study, angau muda is the stage where the sago palm content the highest starch of 41.3% and 41.4%, base and height respectively. This suggests that at flowering stage is the best time for harvesting and most of the starch can be found in the pith of the palm and typically
contain about 250kg of starch (Flach, 1983). After sago palm matured and produced fruits, the palm soon die (Dransfield, 1997).

Sago palm has been identified as highly efficient energy crop that can produce large amount of starch and yielding more ethanol per hectare than any other grown biofuel crops (Biofuel new, 2008). Sago can produce high starch content amounting to about 25t/ha/year by four time greater than rice, corn and wheat (Ishizaki, 1997). In fermentation process, a ton of glucose can be converted to 500kg of ethanol, and so 1000ha of sago plantation can potential generated 12500 ton of biofuel (Bujang et al., 2000). Sago palm has very high photosynthetic ability to accumulate large amount of starch in its trunk and reached at maximum at 15 years old throughout its cycles (Biofuel new, 2008). Hence so, sago palm is a great interest for use as starch sources in ethanol fermentation.

2.3 Sago starch

Typical starch in nature exists in inert granular structure form which composed of macromolecules arranged in polycrystalline state. The granules of starch were insoluble in water and resistant to many enzymes and chemicals action which by heating in water by process of gelatinization will disrupt the granular structure and enhances its chemical reactivity toward hydrolytic enzyme (Wang et al., 1995). Sago starch have many purposes that it can be used in many field such as in traditional way where Melanau race in third division of Sarawak use as staple food (Sim, 1986), making keropok of shrimp cracker (Sidawag & Balasingam, 1971; Ong, 1979), and tebaloi biscuits (Anon, 1980). In food industries, sago starch used as thickener in soup, baby food and additives in food product (Chularatnatol, 2002; Zulpilip et al., 1991; Takahashi, 1986; Ngudiwaluyo et al., 1998). Biotechnology field used sago starch to produce ethanol and gasohol (Pranamuda et al.,

2.4 Yeast: *Saccharomyces cerevisiae*.

The *Saccharomyces cerevisiae* are unicellular fungi and often used in research because of its versatility to be manipulated and cultured, and widely used in industrial applications especially in fermentation to produce alcohol and lactic acid under anaerobic conditions. They grow rapidly and have simple nutritional requirements. Since prehistoric times, yeast has been used as an agent responsible for fermentation of wine, beer, and other alcoholic beverages, and mostly used in the baking of bread (Erland et al. 2006). The performance of yeast in the fermentation process was limited by the ethanol toxicity and tolerance of yeast cell toward it. Casey et al. (1983) suggested that nutritional deficiencies are responsible for the incomplete glucose fermentation rather than ethanol toxicity and the effect of the action of additives been recognized to the enhancement of resistance of yeast cell to ethanol. Soya flour has been identified in many researches such that it enhances the fermentation rate, the amount of sugar consumed, and the final ethanol concentration (Bajpai et al. 1988, Thomas et al. 1994, Li, 1995).

The lipids and proteins contained in the soya flour can assimilate into yeast cell and enhance the ethanol tolerance of yeast where the yeast cell wall is able to absorb the toxic compounds from fermentation media and thus stimulate the ethanolic fermentation (Bafrnova et al., 1999). Previous study by Viegas et al. (1985), observed that there is significant increasing amount of glucose fermentation and high final concentration of ethanol when 4% (w/v) soya flour was added to fermentation media. The presence of soya
flour increases in the rate and extent the yeast growth. The test of 18 to 24 hours fermentation for both supplemented and unsupplemented media with soya flour, it was observed that high viable yeast concentration in supplemented media when the growth stop (Casey, Magnus and Ingledew, 1983). Soya flour was reported to be good yeast nutrient supplement because it can maximize the rate of the glucose consumption and ethanol production (Viegas et al. 1985, Bajpai et al. 1988, Thomas et al. 1995, Li, 1995). The soya flour allowed more and extends growth of yeast because growing yeast at log phase ferment rapidly (Viegas et al., 1985).
3.0 MATERIALS AND METHODS

3.1 Source of materials

*Saccharomyces cerevisiae* CSI-1 (Cirilo-Shimazaki-Ishizaki-1) strand were obtained from Biofuel Research and Development Laboratory of Faculty Resource Science and Technology, Universiti Malaysia Sarawak. The yeast is originally from the Japanese Collection of Microorganism (JCM-15097).

The sago starches were obtained from Biofuel Research and Development Laboratory of Faculty Resource Science and Technology, Universiti Malaysia Sarawak. Industrial grade sago starch is originated from Nitsei Sago Industries, Kampung Teh, Mukah, Sarawak. The industrial sago starch obtained contains 15% moisture.

The soya flour that was used as a supplement in the fermentation is an organic soya, Melilea’s brand label. It is made from non GMO whole organic beans that high in protein and carbohydrate.

3.2 Preparation of Yeast for inoculums

The yeast’s stock culture was initially grown in liquid medium containing glucose as a carbon source and nutrient broth in universal bottles and grown at 30°C in incubator for 24 hours and subculture every 24 hours for maintaining the yeast. After 24 hours, the yeast was transferred to fresh media to be used as inoculums for the fermentation. About 10% of inoculums were used. The inoculums of yeast were prepared 24 hours before the fermentation starts. The yeast inoculums were grown in incubator shaker for 12 hours for
the first inoculums and then transferred about 10ml to new 70ml inoculums media as second inoculums. This was done in order to make the yeast more active in consuming the glucose.

3.3 Preparation of hydrolyzed Sago starch (HSS)

The sago starch was converted into glucose by process of enzymatic hydrolysis by double boiling method. The starch first is liquefied using Termamyl-120L (0.5 µl/kg) at temperature of 90°C at pH 6.5 for 2 hours and follow by saccharification using Dextrozyme at (0.65 µl/kg) at temperature of 60°C at pH 4.5 for another 24 hours (Bujang et. al, 2000). The concentrations of HSS used in this study were 300g/L. Since the sago starch provided contains 15% moisture, the wet weight of sago starch must be weight to obtain dry weight of 300g is by equation 1:

$$Wet\ weight = \frac{Dry\ weight}{0.85}$$  \hspace{1cm} Eq. 1

3.4 Fermentation process

One liter fermentor was used with 700ml of working volume. The concentration of HSS used was 300.6g/L and 296.525g/L for unsupplemented and supplemented of soya flour respectively. About 10% of inoculums of yeast were prepared for the fermentation. The fermentation process was operating about 72 hours. About 10ml of samples were taken from the fermentor for every 3 hour for the first 12 hours, 6 hours for the next 36 hours and finally every 12 hours for the last 24 hours. For fermentation, two different media were set
up where first medium act as a control (without supplemented of soya flour) and second medium supplemented with soya flour. About 4% w/v of soya flour were added. The control was applied to determine the effect of soya flour on yeast viability and their performance. The pH is controlled by pH controller using 5M of NaOH to maintain the medium pH between pH 5.0 to 6.0. At the same time, the temperature was set between 27°C to 30°C to achieve maximum ethanol production for VHG HSS (Jones and Ingledew, 1993). The fermentation medium only contains HSS glucose, nutrient broth and soya flour.

3.5 Analysis

The samples of ethanol and sugar concentration were taken periodically and aseptically during batch fermentation for analysis. Concentrations of biomass were measured by the optical density (OD) using spectrophotometer at 630nm. The OD measure of the yeast is between 0.2-0.5. High Performance Liquid Chromatography (HPLC) system was used to analyze the concentration of glucose and ethanol in the culture broth. A Shimadzu (Kyoto, Japan) chromatographic system that consists of Shimadzu LC-20AT (four pumps) and Shimadzu RID-10A Refractive Index Detector will be used to perform the HPLC analysis. In fermentation monitoring column, the chromatographic separation was performed, Aminex (7.5 mm x 150 mm) at 60 °C. At flow rate of 0.60 ml/min, 0.005 M of H₂SO₄ is used as a mobile phase. In the column, the sample that injected is exactly 20 μl and ethanol standard also used as sample detection with the same amount of condition. The use of ethanol standard for estimate of ethanol equivalent values will give a standard curve.
3.6 Statistical analysis

All the data obtained were calculated for the mean and standard deviation. One-way ANOVA was used to analyze the data and value less than 0.05 was considered significant. Significant data was analyzed with F-distribution to compare means between treatments. F-statistic more than F-critical value considered significant different. Significant difference between treatments was obtained if p value is less than 0.05.
4.0 RESULT AND DISCUSSION

4.1 *Saccharomyces cerevisiae* CSI-1 growth rate

![Figure 1 The growth curve of *Saccharomyces cerevisiae* CSI-1 during batch fermentation under Very High Gravity. Symbols (○) Without soya flour; (●) With soya flour](image)

The time profiles of the OD above showed a growth curve OD of *Saccharomyces cerevisiae* CSI-1 during batch fermentation under very high gravity with and without supplemented of soya flour (control) conditions. It was observed that there were no lag phase was observed after the yeast was inoculated into the VHG medium at both of the experiment. This finding indicated that the HSS did not contain any substances that could inhibit the growth of the yeast cell (Laopaiboon, 2010). At the same time, this showed that the yeast have good adaptation to the new environment and high concentration of sugar since the yeast showed an exponential growth until 18 hours for both of the fermentation condition.
Meanwhile, under control condition, the stationary phase of yeast is longer by 18 hours compared to that supplemented medium. After 48 hours, the yeast OD somehow decreased more than half, from OD25.82 at 48h to OD14.57 at 60h. The yeast in media without soya flour has less tolerance to the high concentration ethanol and that cause many yeast cells died. Compared to the VHG with soya flour, the yeast OD also decreased yet slower but much earlier after 30h. The presence of soya flour might have affected the growth pattern of the yeast. Soya flour contains a lot of proteins and lipids which can be assimilated into yeast cells and enhance ethanol tolerance of yeast (Bafrcova, 1999). This mean that when the ethanol concentration is high and start to create toxicity environment, the yeast cell wall absorb and assimilated the soya flour. From experiment done by Xiao et al (2008), they found, under microscopic observation that soya flour can retain the cell shape while dramatic elongation of cell we observed with the deffated soya flour supplemented medium on cassava. This showed that yeast faced a death phase due to the high concentration of ethanol when the concentration of ethanol reached 100g/L. At the end of the fermentation, the OD of yeast of supplemented media showed higher number than the control. However statistical analysis showed that there is no significant different of the yeast’s growth in unsupplemented and supplemented soya flour medium since F statistic value is less than F critical value.
4.2 Fermentation by *Saccharomyces cerevisiae* CSI-1 under two different supplements

![Graph showing fermentation profile](image)

**Figure 2** Batch fermentation profile of ethanol production from VHG medium without soya flour supplement
Symbol (■)Glucose; (▲) Ethanol

![Graph showing fermentation profile](image)

**Figure 3** Batch fermentation profile of ethanol production from VHG medium with soya flour supplement
Symbol (■)Glucose; (▲) Ethanol

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The fermentation and production of ethanol by *Saccharomyces cerevisiae* CSI-1 was examined. The supplemented of soya flour in the VHG fermentation using HSS as glucose source was carried out to investigate the ability of *Saccharomyces cerevisiae* CSI-1 strain in fermentation process to produce high yield of ethanol. Figure 2 presented the graph of the VHG fermentation without supplemented of soya flour. Meanwhile Figure 3 presented the graph for VHG fermentation with supplement of soya flour. Both graphs show the time profiles of residual total sugar and ethanol concentration during batch fermentation under VHG condition. Based on both graph, it was observed that the yeast is efficient and productive enough in the fermentation since it's utilized almost all the glucose available, and produce high concentration of the ethanol.

For Figure 2, the total sugars at the beginning of the fermentation were 300.6g/L and the value decrease approximately about 40% at 6 hours of the fermentation. About 120g/L glucose were consumed by the yeast. The production of ethanol was low during this period although the sugar consumption rate by yeast is high where only about 19g/L of ethanol produced. The same things also can be interpreted from graph at Figure 3 where the total sugar decreased by 36%, from the initial sugar of 296.52g/L to 189.60g/L. About 107g/L glucose were consumed. This was because the sugar available was used by the cells in their biomass production. It is corresponded to the growth curve of the yeast where the log phases shown. In contrast, the supplemented HSS media show higher production of ethanol when compared to unsupplemented HSS media. At 6 hours, the ethanol concentration for supplemented media was about 30g/L. This might due to the adaptation of the yeast strain to the high concentration of glucose. The yeast takes up the glucose only for growth and multiplication and probably under aerobic respiration where little amount of ethanol only being produced for both fermentation.
The graph both shows the inversely proportional relationship between the concentration of glucose and ethanol, where the decreasing of the glucose result in the increasing production of ethanol. The final concentration of ethanol produced by yeast for control medium was approximately 109g/L and about 26.4g/L of glucose was remained at the end of the fermentation. Meanwhile, for soya flour supplemented medium, the final concentration of ethanol produced was approximately 121g/L and about 8g/L of glucose remained. The final ethanol product and the glucose residue between this two media showed that the presence of soya flour in the medium affect the viability of the yeast. Both of the graphs also provide information that the yeast actively produced ethanol for the 48h period and for the successive 24 hours, there only a little amount of ethanol produce; control batch produced about 16g/L ethanol and supplemented batch produced about 12g/L ethanol between 48h to 72h fermentation. This showed that the yeast cannot stand the toxicity when the ethanol concentration reached 100gL\(^{-1}\) and most cells start to die and decrease in biomass.