PRODUCTION OF BIOETHANOL FROM TAPIOCA STARCH USING *Saccharomyces cerevisiae*: EFFECT OF INOCULUM CONCENTRATION AND TEMPERATURE

MUHD BASHORI BIN ABU BAKAR

UNIVERSITI MALAYSIA PAHANG

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PRODUCTION OF BIOETHANOL FROM TAPIOCA STARCH USING *Saccharomyces serevisiae*: EFFECT OF INOCULUM CONCENTRATION AND TEMPERATURE

MUHD BASHORI BIN ABU BAKAR

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

May 2008

I declare that this thesis entitled “Production of Bioethanol from Tapioca Starch Using *Saccharomyces cerevisiae*: Effect of Inoculum Concentration and Temperature” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Special Dedication to my family members,
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

The inoculum concentration of *Saccharomyces cerevisiae* yeast and the temperature on ethanol production were studied. The optimum conditions of starch hydrolysis such as substrate and enzymes concentration, pH and the time required for the enzymatic action were fixed during the entire process study. The two-step enzymatic hydrolysis of tapioca by commercially available α-amylase and glucoamylase were employed. The fermentation process was run in 250 mL shake flasks. Based on the results, the highest yield of ethanol was 20.7 wt% which was produced at 36°C for the effect of temperature study. However, for inoculum concentration study, at 20% (v/v), the yield of ethanol was 21 wt% which was the highest.

ABSTRAK

Kepekatan inokulum bagi yis *Saccharomyces cerevisiae* dan suhu bagi proses proses penghasilan etanol telah dikaji. Keaadaan optimum bagi hidrolisis kanji seperti kepekatan substrat dan enzim, pH dan masa yang diambil untuk tindakan enzim telah digunakan dalam proses kajian ini. Selain itu, dua langkah bagi proses hidrolisis enzim oleh α-amylase dan glucoamylase telah digunakan. Proses fermentasi dijalankan di dalam kelalang 250 mL. Berdasarkan data yang diperolehi, kadar etanol yang tertinggi adalah 20.7 wt% yang dihasilkan pada suhu 37°C bagi kajian kesan suhu. Bagaimanapun, untuk kajian kesan kepekatan inokulum, pada nilai kepekatan inokulum 20% (v/v), kadar etanol yang terhasil ialah 21 wt% di mana adalah nilai yang tertinggi.

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LIST OF SYMBOLS/ABBREVIATIONS

Ca^{2+}	-	ion calcium
CO_2	-	carbon dioxide
DE	-	dextrose equivalent
DNS	-	Di-Nitro Salicylic Acid
DP	-	degree of polymerization
EU	-	European Union
Glu.	-	Glucose
g	-	gram
h	-	hour
KNU	-	kilo
LODP	-	leveling off degree of polymerization
mg/L	-	milligram per liter
Mg^{2+}	-	ion magnesium
Min	-	minutes
mL	-	mililiter
Mm	-	megameter
OPEC	-	Organization of Petroleum Exporting Country
v/v	-	volume per volume
v/w	-	volume per weight
w/v	-	weight per volume
w/w	-	weight per weight
µg/mL	-	microgram per mililiter
%	-	percentage
°C	-	degree Celsius
°F	-	degree Fahrenheit
µmol	-	micromole

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ethanol or ethyl alcohol (CH_3CH_2OH) is one of the most versatile oxygen-containing organic chemicals. Ethanol act as a solvent, a germicide, a beverage, an antifreeze, a fuel, a depressant and as a chemical intermediate for other organic chemical. The use of fermentation-derived ethanol or so called bioethanol as an automotive fuel additive to enhance octane and reduce emissions has seen an explosive growth over the last 12 years worldwide (Logsdon, 1994).

World bioethanol production in 2003 was 23 Mm^3 (Berg, 2004). The major world producers are Brazil and United States, which together account for about 80% of the world production. Agricultural raw materials rich in fermentable carbohydrates could be converted to yield the fermentable sugars. Main feedstock for bioethanol production is sugarcane (in Brazil) and corn grain (USA) (Mojović *et al*., 2006).

Basically, major carbohydrate-containing substrates such as wheat, rice and starch are commonly used sources of food for a major population in Malaysia. Therefore, starchy substrate such as tapioca could be exploited for ethanol production. Recently, Aggarwal *et al.* (2001) estimated that the starch content of tapioca powder was estimated to be 95% and moisture content 2%. Moreover, owing to its high carbohydrate content, tapioca provides one of the most efficient sources of starch. This raw material has not yet been fully exploited in highly technical industrial enterprises for ethanol production. Since the use of starch-based raw

materials for ethanol production is not a common practice in Malaysia, it is imperative to optimize the conditions for the economical hydrolysis of the starchy substrate to produce sugars for subsequent fermentation. Starch is a reserve polysaccharide of plant origin, which cannot be converted to sugars easily. Starch saccharification requires prior gelatinization by heat treatment, liquefaction by a-amylase and conversion to sugars by glucoamylase (Aggarwal *et al*., 2001). There are two major processes on converting from rich fermentable carbohydrates materials to ethanol which are enzymatic hydrolysis (from carbohydrates to sugars) and fermentation by microorganisms such (from sugars to ethanol).

1.2 Objective

The aim of this study/research is to determine the optimum conditions of fermentation process for the production of bioethanol from tapioca starch. Hence, the objectives of this research are:

i. To study the effect of inoculum concentration on the production of bioethanol from tapioca starch
ii. To study the effect of temperature on the production of bioethanol from tapioca starch.

1.3 Scope of Study

Bioethanol production will conducted by two-step process; enzymatic hydrolysis followed by fermentation process. The scope for this study is to determine the yield of bioethanol that can be produced from locally available tapioca starch during fermentation process. Various inoculums concentration of *Saccharomyces cerevisiae* yeast and temperature will be investigated. The optimized conditions of starch hydrolysis from literature survey such as substrate and enzymes concentration, pH and the time required for the enzymatic action will be employed during the process study.

1.4 Problems Statement

Due to the diminishing fossil fuel reserves, alternative energy sources need to be renewable, sustainable, efficient, cost-effective, convenient and safe (Chum and Overend, 2001). In the past decades, the production of ethanol has been focused and considered as an alternative fuel for future since fossil fuel is currently depleted (Najafpour *et al*., 2003). Furthermore, the use of ethanol from renewable lingocelluloses resources may improve energy availability, decrease air pollution and diminish CO_2 accumulation. Ethanol is found to be biodegradable, low in toxicity and cause little environment pollution (Azrul, 2006).

Since ethanol has become one of the major sources as an alternative fuel, it is important to investigate on the production of ethanol as that the process will be time-reducing and cost-effective. Earlier developments in the conversion of starch to ethanol involved acid hydrolysis. However the production of by products such as furfural and formic acid has resulted in lower yields of alcohol and inhibited yeast growth. Acid-hydrolysis also caused the degradation of sugars to toxic 5-hydroxymethylfurfural resulted in the undesirable off-flavours. Moreover, it is not possible to achieve dextrose equivalent (DE) greater than 55 without generation off-taste. Therefore, most acid-hydrolysis has been replaced by enzymatic hydrolysis (Aggarwal *et al*., 2001).

Basically, most of the raw materials used for the production of bioethanol were corn grain and sugar cane (Mojović *et al*., 2006). However, it is also important to see the potential of the other agricultural raw materials rich in fermentable carbohydrates such as tapioca since it is available in Malaysia and cheaper compare to the others.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The demand for ethanol is increasing in recent years because of its wide use in chemical, potable and motor-fuel industries (Suresh *et al*., 1999). Research and development efforts directed toward commercial production of ethanol as the most promising biofuel from renewable resources have increased (Mojović *et al*., 2006). In many developed countries in Europe and in USA, the use of bioethanol as an alternative fuel or a gasoline supplement in the amounts up to 15% is highly recommended or even required as an ecologically favorable fuel oxygenates (Burnes *et al.,* 2005). Concerning the European Union (EU), a new directive was accepted in November 2001 that requires of members states to establish legislation about utilization of fuels from renewable resources. In 2005, this utilization should cover 2% of the total fuel consumption. This quota is expected to increase to 5.75% in 2010 and furthermore. Some member states like Finland, Sweden or Austria have already fulfilled this quota (Berna, 1998).

Utilization of starch and cellulose substrates for ethanol production is now preferred for economic reasons (Suresh *et al.*, 1999). An important issue regarding the bioethanol production is weather the process is economical. Research efforts are focused to design and improve a process, which would produce a sustainable transportation fuel. A low cost of feedstock is a very important factor in establishing a cost effective technology (Mojović *et al*., 2006).

2.2 Background of Ethanol

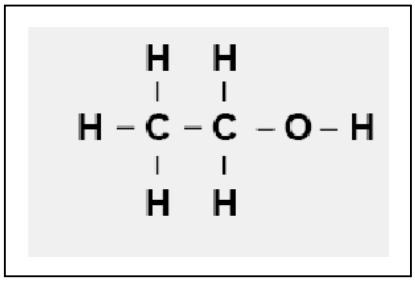

Figure 2.1: Ethanol Structure

Ethanol (Ethyl Alcohol or Grain Alcohol, C_2H_5OH) can be defined as clear, colorless flammable oxygenated hydrocarbon with boiling point 173.5°F in the anhydrous (Figure 2.2) (Azrul, 2006). Ethyl alcohol is well known as a constituent of alcoholic beverages. As a beverage ethanol had been prepared and used long ago by the Egyptian pharaohs. Some indication of the antiquity of the knowledge of ethyl alcohol is the fact that Noah is believed to have built a vineyard in which he grew grapes that he fermented into a type of alcoholic beverage (Logsdon, 1994).

2.3 Production of Ethanol

Industrial ethyl alcohol can be produced synthetically from ethylene or by the fermentation of sugar, starch or cellulose.

2.3.1 Synthetic Process

There are two main processes in the synthesis of ethyl alcohol from ethylene. The earliest to be developed was the indirect hydration process, variously called the strong sulfuric acid-ethylene process, the ethyl sulfate process, the esterification-hydrolysis process, or the sulfation-hydrolysis process. This process is still use in Russia. The other synthesis process, designed to eliminate the use of sulfuric acid

and which, since the early 1970's, has completely supplanted the old sulfuric acid process in the United States, is the direct hydration process. This process involves the catalytic vapor-phase hydration of ethylene. There are other synthetic methods that have been investigated but have not become commercial. These include, for example, the hydration of ethylene in the presence of dilute acids (weak sulfuric acid process); the conversion of acetylene to acetaldehyde, followed by hydrogenation of the aldehyde to ethyl alcohol (Logsdon, 1994).

2.3.2 Fermentation Process

Fermentation is one of the oldest chemical processes known to man. It is used to make a variety of products, including fuel, foods, flavorings, beverages, pharmaceuticals and chemicals. At present, however many of the simpler products such as ethanol are synthesized from petroleum feedstocks at lower cost. Ethanol production by fermentation, excluding that for beverages, had been declining in United States since synthetic ethanol was introduced in the 1930s, because of the low cost and assured availability of ethylene. The quadrupling of the selling price of crude petroleum by the Organization of Petroleum Exporting Countries (OPEC) in 1973 had a profound impact on fermentation processes for producing ethanol. Furthermore, the unstable price and the availability of crude petroleum had caused the fermentation become an alternatives process to produce ethanol (Logsdon, 1994). Recently, Baras *et al*. (2002) had reported around 60% of the ethanol is produced by fermentation the major world producers are Brazil and the US, which together account for about 80% of the world production.

2.4 Raw Materials for Ethanol Fermentation

Ethanol can be derived by fermentation processes from any material that contains sugar or compounds that can be converted to sugar. The many and varied raw materials used in the manufacture of ethanol via fermentation are conveniently classified under these three types of agricultural raw materials: sugar, starches and cellulose materials. Sugar (from sugar cane, sugar beets, molasses, or fruit) can be converted to ethanol directly. Starches (from grains, root crops) must first be hydrolyzed to fermentable sugars by the action of enzymes from malt or molds. Cellulose (from wood, agricultural residues or waste sulfite liquor from pulp and paper mills) must likewise be converted to sugars, generally by the action of mineral acids. Once simple sugars are formed, enzymes from yeast can readily ferment them to ethanol (Logsdon, 1994).

2.4.1 Sugars

The direct fermentation of sugar cane juice, sugar beet juice, beet molasses (by-product in the production of beet sugar), fresh and dried fruits, cane sorghum, whey and skim milk had been considered as a means of obtaining ethanol. However, none of these raw materials could compete economically with molasses. Although the manufacture of ethanol from the sugar- containing waste products of the fruit industry appears to be a highly desirable operation, particularly as a means of reducing stream pollution in the vicinity of canning plants, such production is costly because of the need to remove most of the water (as much as 97%) contained in the waste product (Logsdon, 1994).

2.4.2 Starches

Starch occurs naturally in most plant tissues, including roots and tubers, cereal grains, vegetables and fruits. The principal components of starch are amylase and/or amylopectin. Amylose is an essentially linear polysaccharide composed of (1-4)-linked α-D-glucopyranosyl units. Because of its helical structure, amylase is able to complex with hydrophobic molecules. Complexed amylase molecules retrograde less effectively. Hence, molecules complexed with hydrocarbon chains provide greater stability to foods. Amylopectin has a branch-on-branch structure. Amylopectin molecules are composed of chains of (1-4)-linked α-D-glucopyranosyl units; branches are formed by joining these chains with α-D-(1-6) linkages. The average chain length is 20 to 30 units, although branch points are not equally spaced (BeMiller, 2000).

Fermentation of starch from grain is somewhat more complex than fermentation of sugars because starch must first be converted to sugars and then to ethanol. Starch is converted enzymatically to glucose either diastase present in sprouting grain or by fungal amylase. The resulting dextrose is fermented to ethanol with the aid of yeast, producing CO_2 as a coproduct. Other by-product depends on the type of process (Logsdon, 1994).

2.4.3 Cellulosic Materials

The technology for converting the cellulosic materials into ethanol is available, but the stoichiometry of the process is disadvantageous. Even if each step in the process of the conversion of cellulose to ethanol proceeds with 100% yield, almost two-thirds of the mass would disappear during the sequence, most of it as carbon dioxide in the fermentation of glucose to ethanol. This amount of carbon dioxide leads to a disposal problem rather than to a raw material credit.

Starch and cellulose are both polymers of glucose, but cellulose is much more difficult to hydrolyze to the sugar. Its structure is more crystalline which protects the internal bonds from hydrolysis, and cellulose in plants is protected by lignin, a polyphenolic material that forms a seal around the cellulose for further protection against hydrolysis. Cellulosic wastes also contain substantial amounts of hemicellulose, which is a polymer of pentoses. The aqueous mineral acids used to hydrolyze the cellulose to glucose destroy much of the sugars, particularly the pentoses, in the process (Logsdon, 1994).

2.5 Acid Hydrolysis

Initially, acid hydrolysis appears to be a relatively efficient means of accessing and breaking down cellulose. The hydrogen ion, therefore, does not face the problem of accessibility compared to cellulase enzymes. Furthermore, the basic mechanism of the 20 hydrolysis of glycosidic bonds is relatively simple the mechanism is similar to the hydrolysis of other glycosides such as starch (α1-4 linked glucose chains, with α1-6 branches). Step 3 is the rate-limiting step of the process because of the formation of the high energy half-chair configuration by the cyclic carbonium ion. Initial hydrolysis rates are typically very rapid performed experiments to show that in the initial stages of the hydrolysis reaction, larger pore volumes do correspond to faster reaction rates. However, after limited hydrolysis, the reaction rate slows down considerably. The glycosidic bonds most susceptible to hydrolysis are those either at the surfaces or in the amorphous regions of cellulose. Rapid hydrolysis rates reflect hydrolysis activity in these regions and can be seen as a decrease in the degree of polymerization (DP) from several thousand to about 200. This point is referred to as the leveling off degree of polymerization (LODP). Further hydrolysis is much more difficult beyond the LODP because of the high crystallinity of the remaining cellulose molecules (Azrul, 2006).

This technique is practiced on commercial scale for glucose production from cellulose. The operation is carried out at an elevated temperature and glucose production efficiency by this process goes up to 50%. However, yield reductions

inherent in glucose degradation during dilute acid hydrolysis at high temperatures are not present in concentrated acid hydrolysis at lower temperature. This process depends on disruption of the crystalline structure of the cellulose by solution or swelling in the acid. The cellulose can then be rapidly hydrolyzed at low temperature to avoid degradation, making almost quantitative yields of glucose attainable. However, in the process, high capital cost is unavoidable because of expensive corrosion resistant equipment, acid recovery plants and higher operation costs (Ajit and Basant, 2003). Moreover, one of the major problems with hydrolyzates produced by acid hydrolysis is the poor fermentability caused by the presence of inhibitors in the hydrolyzates. Furfural is known to be one of the most important of these inhibitors. It is a breakdown product from pentoses and is formed in a browning reaction during hydrolysis in the presence of strong acids. It therefore may be impossible to completely avoid furfural formation in a chemical hydrolysis process designed to give a high sugar yield (Taherzadeh *et al*., 1999).

2.6 Enzymatic Hydrolysis

Lately interest in the enzymatic hydrolysis to get glucose and ethanol has increased as this involved milder condition. Although enzymatic hydrolysis of starchy substrates can give 100% yield of glucose, the reaction is much slower as compared to acid hydrolysis. But the severe problem (corrosion of reactor) during acid hydrolysis process can be avoided by enzymatic hydrolysis process (Ajit and Basant, 2003). Basically, there are two major processes involve in enzymatic hydrolysis; liquefaction and saccharification. The main role of enzymatic hydrolysis is to effectively provide the conversion of two major starch polymer components (amylose and amylopectin) to fermentable sugar that could subsequently be converted to ethanol by yeast.

2.6.1 Liquefaction

The starch content of tapioca powder was estimated to be 95% and moisture content 2%. Liquefaction under pressurized steam was found to be more effective than that of using water bath at since the slurry of tapioca powder was liquefied in a significantly shorter time. The slurry of 35% consistency, which could not be liquefied within 4 hours under either temperature conditions, was discarded keeping in view the time as one of the important considerations in liquefaction process. A slurry of 25% consistency was found to be more appropriate for this process as the liquefaction took only 45 min at 104°C and 120 min at 95°C. Since the pressurized heat yielded better results, the liquefaction in further experiments was carried out under this condition, keeping the total time requirement of 45 min using 25% slurry of tapioca powder. The shorter period of liquefaction in an autoclave could be due to the uniform heating under pressure and constant maintenance of temperature throughout (Aggarwal *et al*., 2001).

The liquefaction was achieved within 45 min as visualized by starch–iodine reaction. This liquefaction protocol provided additional advantages as the gelatinization and liquefaction steps were carried out in a single-step thus saving the energy incurred for the sterilization of starchy substrates. The optimized concentration of enzyme was found to be 0.15%, v/w. Liquefaction took twice as long on reducing the enzyme dose from 0.15 to 0.10% for 25% slurry. In order to determine the optimum pH for liquefaction, tapioca slurry was prepared in buffer of pH values 5.0, 5.5. 6.0, 6.5 and 7.0, and liquefied using 0.15% enzyme dose. The results of starch–iodine reaction showed that efficient liquefaction of tapioca was achieved in a pH range of 6.5–7.0. To examine the effect of divalent ions on the process of liquefaction, various concentrations of calcium chloride and magnesium sulphate providing Ca^{2+} and Mg^{2+} ranging from 25 to 250 mg/l were added to the slurry. The results obtained suggested a requirement of Ca^{2+} was required for liquefaction process of similar duration in presence of Ca^{2+}. Therefore to reduce the enzyme dose from 0.15 to 0.10% (w/v), the calcium chloride supplementation was optimized and found to be 120 mg/l of Ca^{2+}. At concentrations of calcium lower than

120 mg/l, liquefaction required 0.15% enzyme concentration i.e. 50% more. Magnesium sulphate did not show any effect on liquefaction (Aggarwal *et al*., 2001).

2.6.2 Saccharification

For this step, the concentrated crude preparation of glucoamylase from *Aspergillus sp*. was used. The maximum amount of sugars (up to 90%) was produced after 24 h. Glucose was the main sugar in the enzymatic hydrolysate of tapioca starch as detected by paper chromatography. Maximum saccharification occurred at 60°C, at higher temperature the rate of saccharification reduced substantially. The optimum pH for the saccharification was found to be 5.0. With above optimized conditions for the saccharification (time, temperature and pH), the concentration of glucoamylase was optimized (Aggarwal *et al*., 2001).

The saccharification improved with the increasing enzyme units within the range of 10–30 U/ml. To achieve 92% saccharification, the enzyme was needed at the concentration of 30 U/ml, which was close to the expected value. Higher units did not prove effective. Effect of addition of divalent ions on the process of saccharification was studied by the addition of calcium chloride, magnesium and zinc sulphate to provide these ions in the range of 25–250 mg/l. Results obtained for the level of saccharification in presence of these ions, indicated that irrespective of type and the concentration saccharification was similar in all cases as in control showing no effect of divalent ions. The parameters must be standardized for the type of substrate to be hydrolysed. It seems to be a common practice of producing hydrolysate from dilute slurries and to concentrate low-sugar hydrolysates, dilute slurries requires lesser time for saccharification. In many cases relatively high doses of glucoamylases and other maceration enzymes besides amylases such as xylanase, cellulase and pectinase are necessary to saccharify various starch-containing substrates efficiency. Moreover, the efficiency of an enzymatic starch saccharification process depends on the activity of the glucoamylase and also on the purity of enzyme (Aggarwal *et al*., 2001).

The saccharification was more effective when the tapioca was liquefied under pressurized steam in autoclave. This is due to the decrease in the crystallinity of the granules as intermolecular hydrogen and hydrophobic bonds between double helices of the starch chains are broken during the melting process of gelatinization (Aggarwal *et al*., 2001). Besides, by using enzymes that enhance the action of glucoamylase during saccharification, glucose level can also be increased. Enzymes available for this purpose are pullulanase. Pullulanase is specific for the hydrolysis of α-1,6 glucosidic linkages and, when used in combination with glucoamylase, increases the rate and extent of glucose production (Schenck, 2000).

2.6.3 Two-step Enzymatic Hydrolysis

Most of the enzymatic hydrolysis was done by using simultaneous action of α-amylase and glucoamylase. However, Kimura and Robyt (1995) reported that lower than 80% yields of glucose obtained in one step enzymatic hydrolysis with glucoamylase. Recently, high conversion (DE = 92.1%) was obtained in a two step enzymatic hydrolysis with 36AGU of Supersan within 24 hours performed by Mojović *et al*. (2006). This indicates that two-step enzymatic hydrolysis is more efficient compared to one-step enzymatic hydrolysis. Furthermore, the advantages of this process are lower energy consumption and a lower content of non-glucosidic impurities and thus much better suitability for ethanol production.

2.7 Ethanol Fermentation

Starch hydrolyzates obtained by the two-step hydrolysis of tapioca flour were subjected to ethanol fermentation by *S. cerevisiae* under aerobic conditions (refer to Equation 2.1).

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2\ CO_2 \qquad \text{(Equation 2.1)}$$

It was considered that the pasteurization of the substrate achieved during the enzymatic liquefaction (85.8°C for 1 h) was sufficient thermal treatment, and thus no additional sterilization prior to fermentation was performed. The mashes containing various initial starch concentrations were fermented by yeast up to 48 h in flasks in thermostated water bath with shaking. The effect of initial yeast concentrations was studied by applying different inoculum concentrations. During the fermentation, the consumption of the substrate was followed as well as the formation of ethanol. The experiments with simultaneous fermentation and second step hydrolysis were also performed. Mojović *et al.* (2006) reported that fermentation and second step hydrolysis can be concurrently performed, and no shortage of fermentable sugars observed, suggesting that these two processes were in accordance. In this way, the time for overall process may be reduced for 4 hours (the time required for second hydrolysis step). By concurrent fermentation and hydrolysis, the energy savings could be attained since the overall process was effectively performed at 32°C, which is a lower temperature than the optimum temperature for the action of glucoamylase itself (55°C).

Mojović *et al*. (2006) also found that the increase in inoculum concentration did not have pronounced effect on the final ethanol concentration. However the final ethanol concentration still increases when increasing the inoculum concentration. The final ethanol yields obtained by fermentation with 1, 1.35 and 2% (w/w) of inoculum were 78.5, 80.1 and 81.6% of the theoretical yield, respectively. Yet, the duration of fermentation decreased with the increase of the inoculum concentration. For the effect of temperature to the ethanol yield, Rivera *et. al.* (2006) reported that even small changes in temperature affect the kinetics, and, thus, productivity and conversion. Based on the study conducted, the results show the increasing in ethanol yield when the fermentation was performed in the temperature range 28-40°C. Conversely, at 40°C, the ethanol cannot be produced because the temperature exceeds the limit (Rivera *et. al.*, 2006).

CHAPTER 3

METHODOLOGY

This chapter will discuss about the process for producing ethanol through enzymatic hydrolysis and also fermentation process. In this study, the fermentation process will be run by using Shake flask 250 mL in aerobic condition. Finally the reducing sugar analysis, turbidity analysis to measure the cell growth, cell concentration and also ethanol determination will be showed as analysis procedures in this experiment.

3.1 Starch

Tapioca flour was purchased from the local market and used as the starchy substrate for the production of sugar.

3.2 Microorganisms

The microorganism used for the fermentation of tapioca starch is *Saccharomyces cerevisiae*.

3.2.1 Agar Preparation

Agar plate containing (per liter): 50g of glucose, 5g of yeast extract, 2g of KH_2PO_4 ,1g of $MgCl_2$, 1g of $NH_4Cl_2.H_2O$, 15g of nutrient agar and maintaining the pH at 5.5. A 100 ml of agar solution is prepared in a 500 ml Schott bottle. The Schott bottle is filled with distilled water until 100 ml. The solution of agar plate is poured into the Schott bottle. Next, the bottle is shaking until the solution is mixed well. The solution in the bottle was sterilized for 15 minute at 121^{o}C (Noranisah, 2005). The agar solution is poured into different petri dishs. Each of the petri dishs were capped then held at slanted position until it hardens. After that the petri dish are kept in refrigerator at 6˚C-8 ˚C (Noranisah, 2005).

3.2.2 Liquid Medium of Batch Fermentation

A seed culture of *Saccharomyces cerevisiae* is grown in liquid medium of batch fermentation containing (per liter): 100g of glucose, 10g of yeast extract, 4g of KH_2PO_4 ,2g of $MgCl_2$, 2g of $NH_4Cl_2.H_2O$ and maintaining the pH at 5.5. A 1000 ml of liquid medium solution is prepared in Schott bottle. Then, the bottle is hand shake to mix the solution and the media is sterilized for 15 minute at 121^{o}C (Noranisah, 2005). After that the bottles are kept in refrigerator at 6˚C-8 ˚C (Noranisah, 2005).

3.2.3 Culture Maintenance

Culture maintenance of *Saccharomyces cerevisiae* is done in two different ways, which is in single colony unit form on agar plate and in liquid medium and kept in refrigerator at 4^{o}C as a stock culture. 10 % (v/v) of stock culture is grown up in non-oxygen liquid medium and kept it at 35^{o}C for 72 hours. Single colony unit of *S. cerevisiae* is done by streaking the yeast from the solution on to agar plate surface using the inoculating loop in a laminar flow cabinet to prevent any contamination.

All the petri dishs are kept at 35 ^{o}C for 72 hours in aerobic condition. The agar plate which has a single colony unit on agar surface will keep as a stock.

3.2.4 Inoculum's Preparation with Optical Density (OD) 0.5

There are two stages of inoculum's preparation. First is yeast activation stage and secondly is inoculum's producing stage. The activation stages is done by transfer the single colony unit of yeast from the agar plate into the fresh liquid medium of batch fermentation and keep in anaerobic condition at 35^{o}C for 30 hours. Then followed by the second stage, inoculum's producing stage where 10%, 15% and 20% (v/v) of activation culture is inoculated into fresh liquid medium of batch fermentation. During the transferring process, the inoculating loop is flamed with a Bunsen burner until it is red hot in order to sterilize it. The test tube cap that contains the stock culture is removed and the lip of the test tubes is flamed using the Bunsen burner. The inoculating loop is inserted to pure culture test tube and a small portion of the pure culture was removed. After that, the test tube lip is flamed again and thereafter closed tight. One of the serum bottles is used as controller. All the procedures outlined above are done in a laminar flow cabinet to prevent any contamination. The bottles then are incubated in Double Stake Shaking Incubator at 30^{o}C and 1500 rpm for 18 hours (Noranisah, 2005). The bottle is took out from the incubator and kept in refrigerator at 6˚C - 8 ˚C (Noranisah, 2005).The optical density for inoculum was measured by using UV-visible spectrophotometer at 600nm.

3.3 Enzymes

Termamyl 120L, a heat-stable α-amylase from *Bacillus licheniformis* will be used for tapioca flour liquefaction. This enzyme activity is 120KNU/g (KNU, kilo novo units α-amylases- the amount of enzyme which breakdown 5.26 g of starch per hour according to Novozyme's standard method for the determination of α-amylase). Supersan 240L *Aspergillus niger* glucoamylase, activity 240AGU/g (AGU is the

amount of enzyme which hydrolyses 1 μmol of maltose per minute under specified conditions) will be used for tapioca flour saccharification (Mojović *et al.*, 2006).

3.4 Preparing Acetate Buffer (pH 4.8)

A 1L Erlenmeyer flask is filled up with distilled water to 1 L. Sodium acetate anhydrous is weighed for 16.4-gram in a weighing boat and poured inside the Erlenmeyer flask. The solution is stirred until all the powder dissolved in water. Next, 11.5ml of glacial acetic acid poured in a clean measuring cylinder. That contains 988.5mL distilled water. The solution is stirred until it mixed well. Measure the pH using the pH meter until reaches 4.8 value. This buffer solution is prepared for another set with same procedure.

3.5 Hydrolysis Experiment

Tapioca flour, 100g was mixed with water to get 25% w/v concentration and 120 mg/L of Ca^{2+} (as $CaCl_2$) ions is added. The mixture was treated with enzymes in two steps. For the first step, the slurry of tapioca flour was liquefied with 12KNU of Termamyl 120L at 104°C for 1 hour in water bath. After finish the first step of hydrolysis, the saccharyfying enzyme, Supercan glucoamylase is added in the amount of 36 AGU for 100 g of tapioca for next 4 hours also in water bath.

3.6 Fermentation Experiment

The mixture is well homogenized and immediately after that inoculated with 10%, 15% and 20% (v/v) of *S. cerevisiae*. The mixture is fermented in aerobic conditions for 48 hours in double stack incubator at 150 rpm. Samples of fermented were taken each 6 hours within 48 hours and centrifuged at 28,000 rpm for 10

minutes with temperature at 25 ^{o}C to remove the cells (Azrul, 2006). Each sample was analyzed to monitor ethanol production as well as sugar consumption.

3.7 Method of Analysis

3.7.1 Preparation of Di-Nitro Salicylic Acid (DNS) Reagent

To determine the glucose in each sample of tapioca**,** the DNS method (Miller, 1959) was performed by measuring the absorbance at 540 nm. A 50 mL of 2 M NaOH solution was initially prepared. 2.5 g of 3,5-dinitrosalicylic acid was added into the alkaline solution resulting an orange color mixture. The mixture was heated and stirred on a hot plate stirrer. In a separated beaker, a 75 g of sodium-potassium tartarate was dissolved in 125 mL of distilled water. The solution was heated to obtain a hot salt solution. Both the hot DNS and salt solutions were then mixed together and stirred continuously. The mixed solution was then cooled down to ambient temperature. Next, the mixture was transfer into 250 mL volumetric flask and was diluted to 250 mL.

3.7.2 Total Reducing Sugar Determination by DNS Method

A 1 ml of sample is taken out and put in a lightly capped test tube. Then 1 ml of DNS reagent is added to each of test tube. The solution was vigorously shaken and later was heated at 90 ^{o}C for 10 min to develop red-brown colour (Noranisah, 2005). After that, the sample is cooled at room temperature in a cold water bath for another 10 min. The solution was later diluted with additional 8 mL distilled water and analyzed for total reducing sugar concentration. The absorbance was recorded using UV-visible spectrophotometer at 540 nm.

3.7.3 Ethanol Determination

The amount of ethanol was determined by measuring the measuring the weight decrease of the system at certain intervals and by using Mettler Toledo in order to determine the weight percent and volume percent of ethanol in each sample. This phenomenon is reflected as a weight decrease, which can be correlated to the amount of the ethanol produced (Noranisah, 2005). In terms of weight, every gram of glucose can theoretically yield 0.51 g of ethanol. It is assumed that 50% of glucose was used to produce ethanol and 50% of it to produce CO_2; thus there is a weight decrease due to the amount of CO_2 removed from the system and the amount of the ethanol that was produced (Noranisah, 2005).

3.8 Preparation of Standard Calibration Curve for Glucose

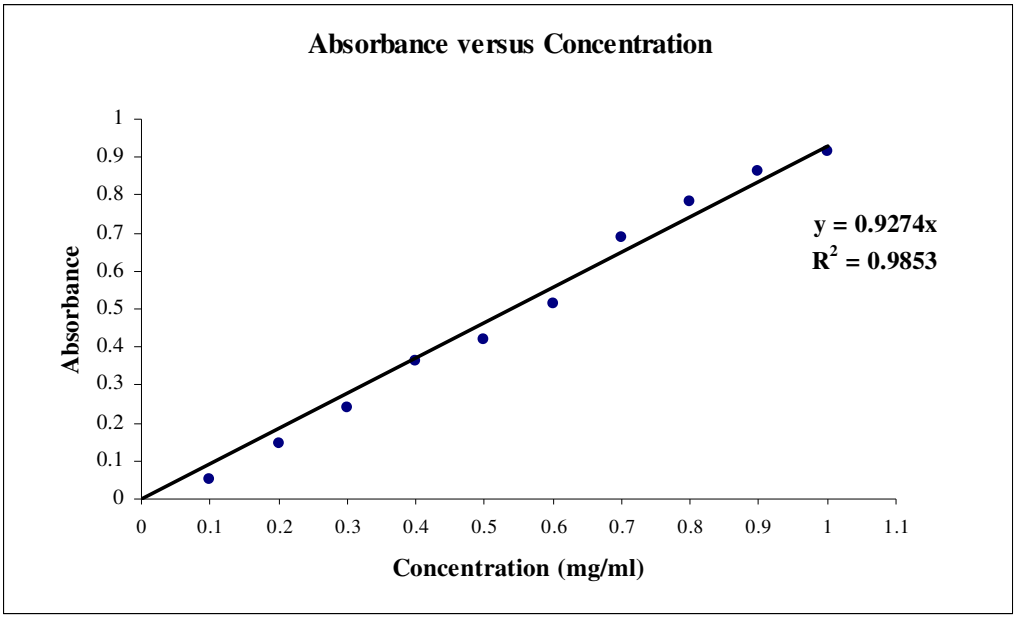

Figure 4.1: Standard Calibration Curve for Glucose

The graph above was constructed based on the data obtained after the standard glucose was tested by using DNS method. This graph was important in order to find the concentration of glucose in every sample being tested. The data for

the standard calibration curve can be found at **Appendix C.1**. This preparation is important in order to find the concentration of tested. A 1000µg/ml standard glucose solution is prepared by adding 100mg of D (+)-glucose anhydrous into 100 ml of distilled water. The solution is stirred until all glucose dissolved in the water. Next, 5 ml glucose solution of 5 different concentrations is prepared from 1000µg/ml glucose solution. The first concentration is 50 µg/ml is prepared by adding 0.25ml of glucose solution into 4.75 ml of distilled water. The procedure repeated for the next 9 concentrations. After all of the glucose solution is ready, 3ml of each glucose solution from different concentration is spurge out using micro Spurger and put it in a lightly capped test tube. Next, all the solution is being test with DNS method.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

In this study of the fermentation process of tapioca starch, the different parameter of inoculum concentration of *Saccharomyces cerevisae* and temperature of fermentation process were studied. The 150 rpm of agitation speed were used as the standard value for all shaking purposes. An aerobic fermentation process was done in batch and was carried out with different temperature; 28, 32 and 36°C. Also for inoculum concentration used was 10, 15 and 20% v/v. The purpose here was to observe the effect of temperature and inoculum concentration on the yield of ethanol produced by the fermentation process.

4.1.1 Effect of Different Inoculum Concentration to Ethanol Yield

To study the effect of inoculum concentration, there were several value of inoculum concentration were employed which were 10%, 15% and 20% v/v. The inoculum age used for this preparation was 18 hours and agitation speed was 150 rpm. During the fermentation process, the temperature used was 37°C. The results for this study are as follows:

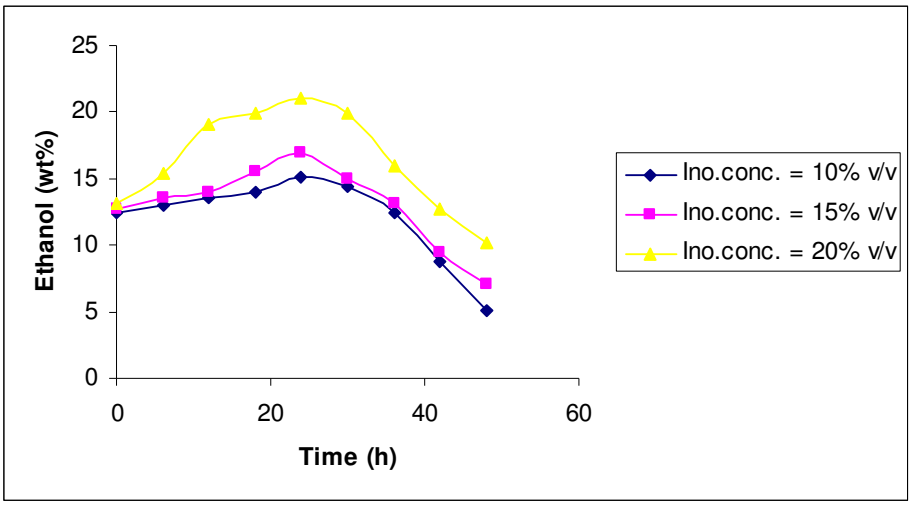

Figure 4.2: The effect of inoculum concentration in ethanol production

Based on the observation from **Figure 4.2**, when the time increases, the ethanol production was increases. The highest ethanol yield produced was 21 wt% which was at 20% v/v. However, when the time exceeds 24 hours, most of the ethanol in the sample decrease. This is due to the decreasing of the carbon sources in the flasks. This observation was similar with Lee *et. al.* (2007). Besides, the initial reading (at 0 hour) for ethanol concentration shows that there was ethanol in the samples. Actually, this situation was a normal thing because during the yeast activation and inoculum preparation, the ethanol was already been produces before the fermentation experiment started.

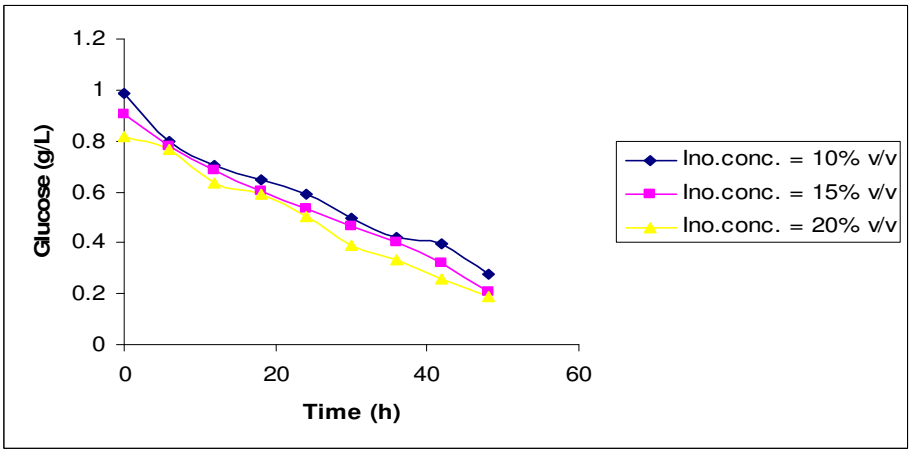

Figure 4.3: The effect of inoculum concentration in reducing sugar

From **Figure 4.3**, when the time increases, the glucose concentration was decreases. The concentration of glucose was constantly decrease during the fermentation since the glucose was consumed by the yeast and converted it to ethanol. The glucose decrease more rapidly when inoculum concentration at 20% v/v compared to 10% v/v and 15% v/v.

4.1.2 Effect of Different Fermentation Temperature to Ethanol Yield

To study the effect of fermentation temperature to the ethanol yield, there were several value of temperature were employed which were 28°C, 32°C and 36°C. The inoculum age used for this preparation was 18 hours and agitation speed was 150 rpm. During the fermentation process, the inoculum concentration used was 20% v/v. The results for this study are as follows:

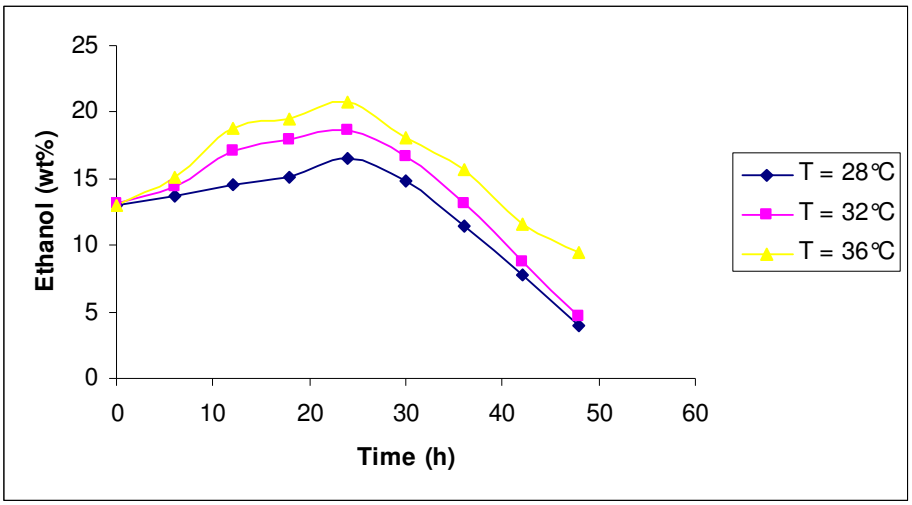

Figure 4.4: The effect of fermentation temperature in ethanol production

Based on the observation from **Figure 4.4**, when the time increases, the ethanol production was increases. The observation was same with the effect of inoculum concentration showed in subchapter before. The higher temperature used, the higher ethanol were produced. The highest yield of ethanol was 20.7 wt% which was produced at 36°C. Based on the results from Mojović *et al.* (2006), the final

ethanol yields obtained by fermentation with 1, 1.35 and 2% (w/w) of inoculum were 78.5, 80.1 and 81.6% of the theoretical yield. This finding was similar with this study.

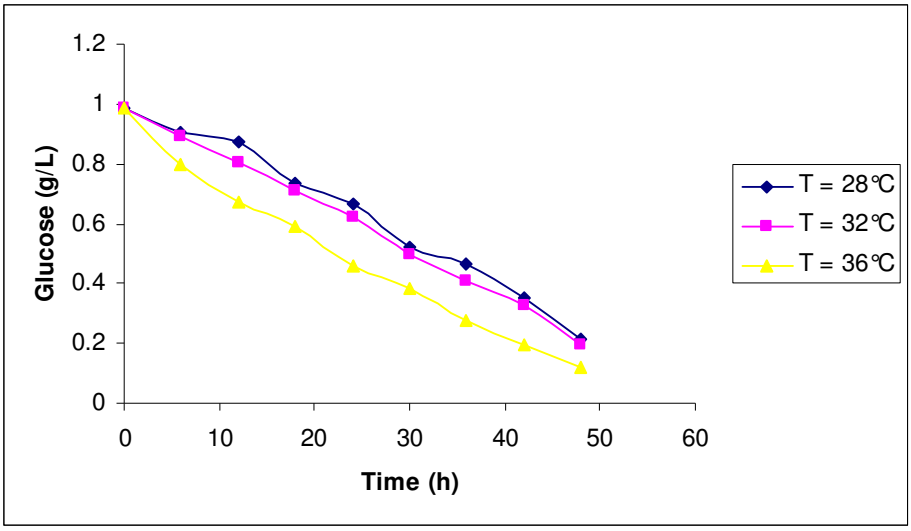

Figure 4.5: The effect of fermentation temperature in reducing sugar

The higher temperature used, the higher ethanol were produced and the concentration of glucose become lower. Here, the temperature at 36°C produces the highest yield of ethanol. Basically, temperature is one of the most important factors in fermentation process. Enough heat supply is the good condition for the yeast to react (Rivera *et. al.*,2006). When the temperature increases, the heat supplied to the yeast increases and this condition was actually can increase the rate of reaction. As a result, the ethanol produce from the fermentation was increases also.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The fermentation process was carried out with pH, agitation speed, time for fermentation and inoculum age was at optimum condition. The effect of inoculum concentration and the temperature in ethanol production were studied. During the enzymatic hydrolysis, α-amylase and glucoamylase were successfully convert tapioca starch into glucose. Based on the observation for the fermentation in aerobic condition, the ethanol production became higher when the temperature and inoculum concentration higher. The highest yield of ethanol produced was at 36°C and 20% v/v of inoculum concentration. Based on the results, the highest yield of ethanol was 20.7 wt% which was produced at 36°C for the effect of temperature study. However, for inoculum concentration study, at 20% (v/v), the yield of ethanol was 21 wt% which was the highest.

5.2 Recommendations

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In order to improve this research, there are several things should be stress out in the future. Firstly, the parameter of study should be increase so that the results can be clearly justified and proved. Furthermore, this process is actually an energy consuming process. It is recommended to use simultaneous hydrolysis and fermentation. Lastly, to improve the enzymatic hydrolysis, use the other kind of enzyme that is more heat stable.

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APPENDIX A

Appendix A.1: UV-Visible Single Beam Spectrophotometer (Model U-1800)

Appendix A.2: Shaking Water Bath (Model BS-21)

Appendix A.3: Double Stack Shaking Incubator Infors

Appendix A.4.: Laminar air Flow Cabinet (Model AHC-4A1)

APPENDIX B

Appendix B.1: Data for standard calibration curve of glucose

Concentration of Glucose (mg/mL)	Absorbance at 540 nm
0.1	0.053
0.2	0.146
0.3	0.241
0.4	0.362
0.5	0.422
0.6	0.516
0.7	0.691
0.8	0.781
0.9	0.863
1	0.913

Appendix B.2: Data for the effect of inoculum concentration in ethanol production.

Time	28°C		32°C		36°C	
	Ethanol (wt%)	Gluc. (g/L)	Ethanol (wt%)	Gluc. (g/L)	Ethanol (wt%)	Gluc. (g/L)
0	13	0.987	13.1	0.988	13	0.988
6	13.7	0.905	14.4	0.891	15.1	0.799
12	14.5	0.876	17.1	0.802	18.8	0.672
18	15.1	0.735	17.9	0.713	19.5	0.593
24	16.5	0.668	18.6	0.622	20.7	0.457
30	14.9	0.521	16.7	0.494	18.1	0.384
36	11.4	0.463	13.2	0.41	15.7	0.279
42	7.8	0.353	8.8	0.328	11.6	0.194
48	4	0.211	4.6	0.197	9.4	0.119

Appendix B.3: Data for the effect of temperature in ethanol production.

Time	10% v/v		15% v/v		20% v/v	
	Ethanol (wt%)	**Gluc. (g/L)**	**Ethanol (wt%)**	**Gluc. (g/L)**	**Ethanol (wt%)**	**Gluc. (g/L)**
0	12.4	0.988	12.7	0.903	13.1	0.814
6	13	0.798	13.5	0.782	15.4	0.766
12	13.5	0.702	14	0.683	19	0.633
18	14	0.647	15.5	0.602	19.9	0.589
24	15.1	0.588	16.9	0.532	21	0.501
30	14.4	0.497	15	0.463	19.9	0.392
36	12.5	0.421	13.1	0.399	16	0.331
42	8.7	0.396	9.4	0.322	12.7	0.258
48	5.1	0.277	7	0.21	10.1	0.191