Production of Ethanol Bio-Fuel from Cassava

by

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Dissertation submitted in partial fulfilment of
the requirements for the
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CERTIFICATION OF APPROVAL

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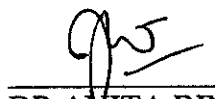
Approved by,



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January 2009

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

ANIS ATIKAH BINTI AHMAD

ABSTRACT

The objectives of this experiment are to know the effect of starch and enzymes concentration towards the glucose production, to observe the texture of starch during the gelatinization process; to study fermentation process of ethanol from Saccharomyces cerevisiae, and to study the rate of ethanol production, by obtaining amount of glucose produced per 1 kg of cassava starch. This study is conducted since there are critical issues of (1) petroleum sustainability, (2) volatility of world's crude oil price; rising significantly that effect all the living costs and (3) the urgent need of alternative, renewable, economic and environmental-friendly fuel. A renewable source, bio ethanol derived from cassava starch is studied as an alternative to petroleum fuel for transportation. The author has studied on starch characterization during gelatinization, and manipulated the concentration of enzymes during hydrolysis. It is found that during the continuous heating, the viscosity of starch keep increasing until a transparent paste is formed, which is a bit sticky. The optimum concentration of enzymes for liquefaction of 20%, 30% and 40% starch slurry is found to be 0.25% α-amylase with 0.2% amyloglucosidase which is similar as reported by Ku Ismail K. S, 2008. The author has proved the study by Aggarwal and Nigam, 2001 regarding the effective period of saccharification which is 24 hours, since the glucose concentration obtained in this experiment is much higher than the results reported by Ku Ismail K. S, 2008, who only saccharified for 1 hour. An improved calibration curve which has greater correlation should be obtained in order to improve the accuracy of the results. Ethanol yield from 20%, 30% and 40% starch with the same amount of enzymes (0.25% α-amylase and 0.2% amyloglucosidase) cassava starch are 14%, 16%, and 20% respectively, which are considered low compared to Seinosuke studies (who obtained around 82.3 and 99.6%). 5 gram of Saccharomyces cerevisiae is assumed to be inadequate for an effective ethanol production. This low production might be due to no supplement provided for yeast, unsuitable environment condition for enhancing yeast growth and inadequate amount of yeast.

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CHAPTER 1
INTRODUCTION

1.1 Background of study

This is a study of ethanol bio-fuel production from cassava. Raw cassava is processed into cassava starch where this cassava starch will then being gelatinized in order to undergo thermal enzymatic hydrolysis. The product of thermal enzymatic hydrolysis is glucose, from reaction of bacterial and fungal enzymes with gelatinized starch through liquefaction and saccharification. The final product, ethanol, is produced from the fermentation of saccharified starch with yeast.

1.2 Problem Statement

Recently the world is shocked by the arising of the crude oil price critically. The crude oil, one of the world's larger demands rose about 74.4% from US$ 74.89 per barrel on 23 July 2007 to US$ 130.63 per barrel on 21 July 2008 (Law KC, 2008). Crude oil price is experiencing extreme volatility, as concerns over supply risk outweighted worries that demand might falter on slowing economic growth worldwide.

Another issue involving crude oil is sustainability. Based on the date given by Energy Information Administration, the crude oil reserve for Malaysia is only 3 billion barrels which is forecast to last another 18 years (Malaysia Energy Data, Statistics & Analysis, Oil, Gas, Electricity, Coal).

Dwindling oil reserves; volatility of world oil prices coupled with problems of supply and dependence; and the urgent need for alternative, renewable, and eco-friendly fuels. These are strong concerns for practically every country in the world, for reasons of self-

sufficiency, and of ecological safety and sustainability. Ethanol is found to be a most satisfactory answer for these concerns

For cassava, the roots of the cassava plant, a major crop in Southeast Asia and other parts of the world, are rich in protein, minerals and the vitamins A, B and C. However, cassava is poisonous unless it is peeled and thoroughly cooked. If it is eaten raw or prepared incorrectly, one of its chemical constituents will be attacked by digestive enzymes and give off the deadly poison cyanide. As little as two cassava roots can contain a fatal dose. According to scientific studies, the bitter variety of cassava contains large amounts of the cyanogenic glycosides "linamarin" and "lotaustralin". Toxic elements are concentrated on its surface or peel. The toxic substances may affect the liver, kidney, and some parts of the brain when the poisons are accumulated in the body. This might be the reason why the Japanese Ministry of Health prohibits the use of cassava for human consumption (Junji Takano, 2008)

With the health effects caused from bitter cassava, it is very suitable to be a feedstock for bioethanol since it is less suitable for human consumption.

1.3 Objective

The objectives of this experiment are; to know the effect of starch and enzymes concentration towards the glucose production, to observe the texture of starch during the gelatinization process; to study fermentation process of ethanol from Saccharomyces cerevisiae, and to study the rate of ethanol production, by obtaining amount of glucose produced per 1 kg of cassava starch.

1.4 Scope of Study

In order to obtain the maximum amount of ethanol from the optimum amount of glucose converted by cassava starch, the author will study about the different concentrations of starch slurries and enzymes needed to yield the maximum amount of ethanol. An optimization of other criteria like pH, amount and type of yeast as well as duration of hydrolysis and fermentation will be also studied.

CHAPTER 2

LITERATURE REVIEW

2.1 Fuel Properties of bioethanol

Bioethanol is a liquid biofuel which can be produced from several different biomass feedstocks and conversion technologies. Production of ethanol from biomass is one way to reduce both consumption of crude oil and environmental pollution. Bioethanol has a higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline. These properties allow for a higher compression ratio, shorter burn time, and leaner burn engine, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (Balat M, 2007). Octane number is a measure of the gasoline quality and can be used for prevention of early ignition which leads to cylinder knocks. Higher octane numbers are preferred in internal combustion engines. An oxygenate fuel such as bioethanol provides a reasonable antiknock value. The anti knock value is the measure of ability to resist detonation. The higher the octane rating, the slower the fuel burns, and the less likely the engine will knock. When ethanol is blended with gasoline, the octane rating of the petrol goes up by three full points, without using harmful additives. Adding ethanol to gasoline "oxygenates" the fuel, adding oxygen to the fuel mixture so that it burns more completely and reduces polluting emissions such as carbon monoxide (Keith Addison, 2008). With 35% oxygen, ethanol, when blended with petrol, causes combustion that is more even and more complete, and reduces knocking or pinging in the motor. The motor runs more smoothly and the overall performance is enhanced.

Summarizing the advantages of ethanol; it is a renewable fuel made from plants which is environmental friendly; manufacturing it and burning it does not increase the greenhouse effect. 10% ethanol blends reduce greenhouse gas emissions by 12-19%; it also provides high octane at low cost as an alternative to harmful fuel additives. Ethanol blends can be

used in all petrol engines without modifications. Ethanol is biodegradable without harmful effects on the environment, it is significantly reduces harmful exhaust emissions. Ethanol's high oxygen content reduces carbon monoxide levels more than any other oxygenate by 25-30%, according to the US EPA. Ethanol blends dramatically reduce emissions of hydrocarbons, a major contributor to the depletion of the ozone layer. High-level ethanol blends reduce nitrogen oxide emissions by up to 20%. Additionally, ethanol can reduce net carbon dioxide emissions by up to 100% on a full life-cycle basis. High-level ethanol blends can reduce emissions of Volatile Organic Compounds (VOCs) by 30% or more (VOCs are major sources of ground-level ozone formation). As an octane enhancer, ethanol can cut emissions of cancer-causing benzene and butadiene by more than 50%. Sulphur dioxide and Particulate Matter (PM) emissions are significantly decreased with ethanol. Ethanol has a positive energy balance, for it generates more energy than is consumed during production. It yields 67% more fossil energy than is used to grow, harvest and process the grain.

The most popular blend for light-duty vehicles is known as E85, which contains 85% bioethanol and 15% gasoline. In Brazil, bioethanol is derived from sugar cane which used pure or blended with gasoline in a mixture called gasohol; containing 24% bioethanol and 76% gasoline (de Oliveria MED, 2005)

2.2 Current Status and Potential Production of Bioethanol

Bioethanol currently accounts for more than 94% of global biofuel production, with the majority coming from sugar cane (International Risk Governance Council). Ethanol currently provides over 40% of the fuel consumed by cars and light trucks (Balat M, 2008). The top ten bioethanol producers are presented in Table 1 (Renewable Fuels Association)

Table 1: Top ten bioethanol producers (billion gallons)

Country	*2004*	*2005*	*2006*
USA	3.54	4.26	4.85
Brazil	3.99	4.23	4.49
China	0.96	1.00	1.02
India	0.46	0.45	0.50
France	0.22	0.24	0.25
Germany	0.07	0.11	0.20
Russia	0.20	0.20	0.17
Canada	0.06	0.06	0.15
South Africa	0.11	0.10	0.10
Thailand	0.07	0.08	0.09

2.3 Feedstocks for bioethanol production

Bioethanol feedstocks can be classified into three types: i)sucrose-containing feedstocks such as sugar beet, sweet sorghum and sugar cane), ii)starchy materials such as wheat, corn and barley; and iii)lignocellulosic biomass such as wood, straw and grasses.(Balat M, 2008). Different feedstocks that can be utilized for bioethanol production and their comparative production potential are given in Table 2.(Linoj Kumar NV, 2006)

Table 2: Different feedstocks for bioethanol production and their comparative production potential

Feedstocks	*Bioethanol production potential (1/ton)*
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse & other cellulose biomass	280

2.4 Cassava in details

Cassava (*Manihot esculenta* Crantz) was originated from Central America (Amazon zone). The plant is known by many names such as cassada, manioc, yuca, tapioca, mandioca, shushu, muk shue, cassave, maniok, tapioka, imanoka, tapioca, maniba, kasaba, katela boodin, manioc, manihot, yucca, mandioca, sweet potato tree, Brazilian arrowroot, and tapioca plant.

Cassava is a tall semi-woody perennial shrub or tree, up to 7 m high, ingle to few stems, sparingly branching; branchlets light green to tinged reddish, nodes reddish. The outer bark is smooth, light brown to yellowish grey; inner bark cream-green; exudates thin, watery; wood soft, creamy straw. The leave is petiole light greenish to red; dark green above, pale light greenish grayish underneath, sometimes variegated; lobes narrow, 2.9-12.5 times as long as wide; central unlobed part usually short, lobes 15-21 times as long. Inflorescences lax, with 3-5 together in fascicles; pedicels light green to red.

The tuberous edible root grows in clusters of 4-8 at the stem base. Roots are from 1-4 inches in diameter and 8-15 inches long, although roots up to 3 feet long have been found. The pure white interior is firmer than potatoes and contains high starch content. The roots are covered with a thin reddish brown fibrous bark that is removed by scraping and peeling. The bark is reported to contain toxic hydrocyanic (prussic) acid, which must be removed by washing, scraping and heating

In general, the crop requires a warm humid climate. Temperature is important, as all growth stops at about 10°C. Typically, the crop is grown in areas that are frost free the year round. The highest root production can be expected in the tropical lowlands, below 150 m altitude, where temperatures average 25-27°C, but some varieties grow at altitudes of up to 1,500 m.

The plant produces best when rainfall is fairly abundant, but it can be grown where annual rainfall is as low as 500 mm or where it is as high as 5,000 mm. The plant can stand prolonged periods of drought in which most other food crops would perish. This makes it valuable in regions where annual rainfall is low or where seasonal distribution is irregular. In tropical climates the dry season has about the same effect on cassava as low temperature has on deciduous perennials in other parts of the world. The period of dormancy lasts two to three months and growth resumes when the rains begin again.

Cassava is drought resistant and grows well in poor soil. It is one of the most efficient producers of carbohydrates and energy among all the food crops.

Cassava is one of the leading food and feed plants of the world. It ranks fourth among staple crops, with a global production of about 160 million tons per year. Most of this is grown in three regions: West Africa and the adjoining Congo basin, tropical South America and south and Southeast Asia. The young tender leaves are used as a potherb, containing high levels of protein and vitamins C and A. The leaves are prepared in a similar manner as spinach, while eliminating toxic compounds during the cooking process.

It is mainly used for human consumption, less for animal consumption and for industrial purposes, though this may vary by country. The roots are rarely eaten fresh but are usually cooked, steamed, fried or roasted when fresh or after drying or fermenting. It is advisable to peel, boil, grind or cut, and dry the roots in order to diminish the contents of cyanogenic glucosides. All plant parts contain cyanogenic glucosides with the leaves having the highest concentrations. In the roots, the peel has a higher concentration than the interior. In the past, cassava was categorized as either sweet or bitter, signifying the absence or presence of toxic levels of cyanogenic glucosides. Sweet cultivars can produce as little as 20 mg of HCN per kg of fresh roots, while bitter ones may produce more than 50 times as much. The bitterness is identified through taste and smell. This is not a totally valid system, since sweetness is not absolutely correlated with HCN

producing ability. In cases of human malnutrition, where the diet lacks protein and iodine, under processed roots of high HCN cultivars may result in serious health problems.

Cassava provides a major source of calories for poor families, because of its high starch content. With minimum maintenance, the farmers can dig up the starchy root of the cassava and eat it 6 months to 3 years after planting. In Africa, people also eat the leaves of the cassava as a green vegetable, which provide a cheap and rich source of protein and vitamins A and B. In Southeast Asia and Latin America, cassava has also taken on an economic role. Various industries use it as a binding agent, because it is an inexpensive source of starch.

Cassava flour is used to make cookies, quick breads, loaf breads, pancakes, doughnuts, dumplings, muffins, bagels. Cassava extracted juice is fermented into a strong liquor called kasiri. The peeled roots of the sweet variety are usually eaten cooked or baked.

The juice can be concentrated and sweetened until it becomes a dark viscous syrup called kasripo (casareep). This syrup has antiseptic properties and is used for flavoring.

Cassava leaves and stem meal are used for feeding dairy cattle. Both fresh and dried cassava roots are consumed by ruminants in different forms (chopped, sliced, or ground). Cassava bushes three to four months old are harvested as forage for cattle and other ruminants. According to recent studies, the leaves of cassava contain a high level of protein, which could surpass those of power foods such as as spinach, love-lies-bleeding or 'kiwicha' (amaranthus caudatus), quinoa (chenopodium quinoa), etc.

Cassava starch is used in the production of paper, textiles, and as monosodium glutamate (MSG), an important flavoring agent in Asian cooking. In Africa, cassava is used as partial substitution for wheat flour.

In Samoa, cassava was used to induce abortion. The Amerindians use the brown juice, obtained during processing, for burns.

Table 3: Typical Composition of Mature Cassava Roots

Moisture	69.8
Starch	22.0
Sugars	5.1
Protein	1.1
Fats	0.4
Fibre	1.1
Ash	0.5

2.5 Processing of cassava to bioethanol

Cassava is a very promising feedstock for ethanol production. There are two types of cassava; bitter and sweet. The bitter type is not edible due to the higher cyanogenic compounds in the roots. Therefore, the bitter type is a suitable source for producing ethanol since it does not compete with food supply.

There are many reasons explaining why cassava has been strongly recommended for bioethanol production. These reasons are applicable to all countries where these plants can be grown and ethanol industry is under consideration. The following reasons are (Klanarong S and Kuakoon P, 2008)

(1) the ease of plantation in various soil types and climate conditions; it can be grown in all types of soil but it prefers loosen-structure soil such as light sandy loams and loamy sands for its root formation
(2) a very low input and investment for planting
(3) "all year round" availability of feedstock as in forms of cheap fresh roots in the harvest season and in dry chips which are readily processed from fresh roots and be stored for uses when the harvest season is over;

(4) a high starch containing raw materials with less impurities. The carbohydrate-rich cassava roots provide a high purity of syrups with a high ratio of fermentable sugars to nonfermentable solids.
(5) a competitive production cost of ethanol from cassava as compared to other feedstock
(6) prospective uses of wastes from ethanol process of cassava as high-value added products.

The cassava will undergo an extraction process to form cassava starch. Starch is composed of two polymers, amylose and amylopectin. Amylose is an essentially linear molecule in which the glucose units are linked through α-1,4 bonds, and it has a double helicle crystalline structure; the helix contains six Ð-glucose molecules per turn. Amylopectin is a highly branched structure with 4-6% α-1,6 bonds at branch points; the average length of branch chain is 20-25 glucose units. It may have a molecular weight in access of 10 (Maiorella, 1985), making it the largest molecule in nature. Starch granules are round or irregular in shape; between 1 and 100μm long. They are held together with internal H-bonds so that they are able to absorb very little water (Aktinson, 1991).

Starch is gelatinized before converted into a sugar by liquefaction saccharification. Starch gelatinization is a process that breaks down the intermolecular bonds of starch molecules in the presence of water and temperature and allows the hydrogen bonding sites (the hydroxyl hydrogen and oxygen) to engage more water. Starch is continuously heated in water until a viscous and transparent texture is formed. As the starch granules being heated, it swells irreversibly and the amylase in the granules become soluble and is released; at this time the viscosity of the gelatinized starch increases dramatically. In order to counteract this and provide a suitable substrate for subsequent saccharification, the starch must be partially hydrolysed or liquefied (Douglas and Colin, 1997).

Liquefaction is a process whereby the concentrated suspension of purified granular starch is converted into solution of soluble, shorter-chain-length dextrins (Douglas and

Colin, 1997). Liquefaction is typically carried out using alpha amylase, an *endo*-acting amylase that hydrolyzes internal 1-4-α-linkages in amylase and amylopectin and rapidly reduces the viscosity of the molecules (Poonam and Dalel, 1995).For liquefaction, a thermostable α-amylase is used which is derived from bacteria like *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. The use of thermostable and acid-stable amylases of microbial origin has been recommended since high temperature and acidic pH are the optimum conditions for the economic hydrolysis of starch.

Liquefaction under pressurized steam (104^0C) is found to be more effective than that of using water bath at 95^0C since the slurry of tapioca powder is liquefied in a significantly shorter time (Table 4). A slurry of 25% consistency is found to be more appropriate for this process as the liquefaction takes only 45 min at 104^0C and 120 min at 95^0C (Aggarwal and Nigam, 2001). The shorter period of liquefaction in an autoclave could be due to the uniform heating under pressure and constant maintenance of temperature throughout. The liquefaction is achieved within 45 min as visualized by starch–iodine reaction.

Table 4: Effect of concentration of tapioca powder in slurry on liquefaction time at different temperatures (Aggarwal and Nigam, 2001)

Tapioca slurry (%, w/v)	Liquefaction time (min)[b]								
	0	30	45	60	90	120	150	210	240
At 104–105 °C (0.3 bar) in autoclave									
15	+	+	−	−	−	−	−	−	−
20	+	+	−	−	−	−	−	−	−
25	+	+	−	−	−	−	−	−	−
30	+	+	+	+	+	+	−	−	−
35	+	−	+	+	−	−	+	+	+
At 95 °C in water bath									
15	+	+	+	+	−	−	−	−	−
20	+	+	+	+	−	−	−	−	−
25	+	+	+	+	−	−	−	−	−
30	+	+	+	+	+	+	+	−	−
35	+	+	+	+	+	+	+	+	+

Starch–iodine reaction: (+) blue colour persists; (-) blue colour disappears

The optimized concentration of enzyme is found to be 0.15%, v/w. The time of 45 min could be reduced to 30 min using a higher dose of 0.30% of α-amylase (Table 5). Rhee et al. (1984) reported faster dextrinization of cassava starch by increasing the α-amylase concentration from 0.10 to 0.40%. The results of starch–iodine reaction showed that efficient liquefaction of tapioca was achieved in a pH range of 6.5–7.0 (Aggarwal and Nigam, 2001).

Table 5: Effect of Biotempase concentration on liquefaction time in tapioca powder slurry (25%, w/v) at 104^0C-105^0C, 0.3 bar (Aggarwal and Nigam, 2001).

Biotempase concentration (%, w/v)	Liquefaction time (30 min)	Liquefaction time (45 min)
0.01	+	+
0.02	+	+
0.05	+	+
0.10	+	+
0.15	+	–
0.20	+	–
0.25	+	–
0.30	–	–

Starch–iodine reaction: (+) blue colour persists; (-) blue colour disappears

Saccharification is the process in which maltodextrins (such as post liquefied starch-containing material) is converted to low molecular sugars; it is carried out in the presence of the carbohydrate-source generating enzyme such as glucoamylase, maltogenic amylase, beta amylase and a combination thereof (Smith and Ress, 2008). Saccharification is also can be defined as a process removing single glucose residues from a soluble oligosaccharide in its simplest form and it is catalysed by an exo-acting 1,4-glucanohydrolase (glucoamylase), which sequentially remove a glucose unit from nonreducing end until all of the oligosaccharide is degraded to glucose (Douglas and Colin, 1997). Fungal α-amylase, glucoamylase or amyloglucosidase, which is derived from a strain of *Aspergillus niger* and hydrolyze consecutive 1,4-α bonds and 1,6-

αbonds in dextrins to yield β-D-glucose (Poonam and Dalel, 1995), is preferably added to completely convert the liquefied starch into glucose. *Aspergillus niger* glucoamylase contains at least two major electrophoretically distinct isozyme forms that differ very little in their enzymatic properties (Meagher M. M and Nikolov Z. L., 1989). The enzyme acts toward a range of glucosidic linkages with the highest affinity to α-1,4-glucosidic bonds (Teague and Brumm, 1992). The ability of the enzyme to hydrolyse α-1,4 and α-1,6-glucosidic linkages provides almost complete starch degradation to glucose.

From Figure 1, saccharification is found to effectively produce maximum amount of sugars (up to 90%) after 24 h (Aggarwal and Nigam, 2001).

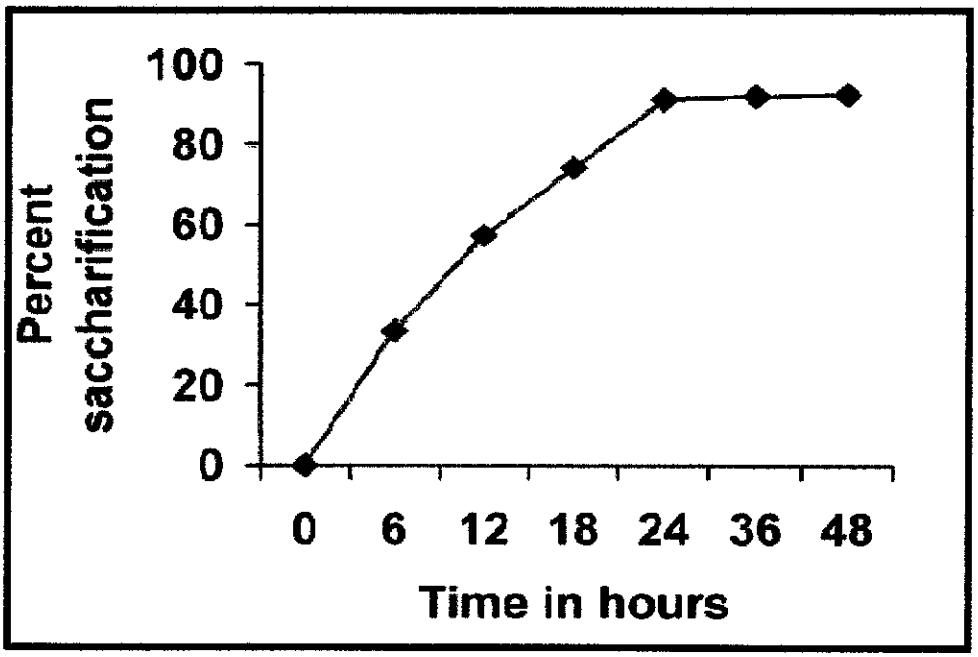


Figure 1: Effect of time on saccharification of liquefied tapioca powder using crude glucoamylase (at 60^0C, pH 5.0, 25%, w/v slurry, enzyme at 30 U/ml liquefied slurry).

Table 6: Effect of temperature, pH and concentration of crude glucoamylase on saccharification of liquefied tapioca powder (Aggarwal and Nigam, 2001)

Temperature (^{0}C)	Saccharification (%)	pH	Saccharification (%)	Enzyme (U/ml)	Saccharification (%)
30	57.37	3.5	55.75	10	41.21
35	61.49	4.0	60.20	20	72.32
45	69.53	4.5	75.53	30	92.52
60	91.47	5.0	91.12	40	90.98
80	49.29	6.0	52.52	-	-

According to the table 6, maximum saccharification is found to occur at 60^{0}C, at higher temperature the rate of saccharification reduces substantially. The optimum pH for the saccharification is found to be 5.0. With above optimized conditions for the saccharification (time, temperature and pH), the concentration of amyloglucosidase is optimized. The saccharification improved with the increasing enzyme units within the range of 10–30 U/ml. To achieve 92% saccharification, the enzyme is needed at the concentration of 30 U/ml, which is close to the expected value. Higher units do not prove effective.

Ku Ismail K. S.(2008) reported that, the optimum concentration of enzymes are 0.25% for α-amylase and 0.15% for amyloglucosidase in a slurry of 30% which produce maximum glucose concentration, 204.5g/l. Figure 2 shows the effect of different enzyme and cassava starch concentration toward glucose concentration.

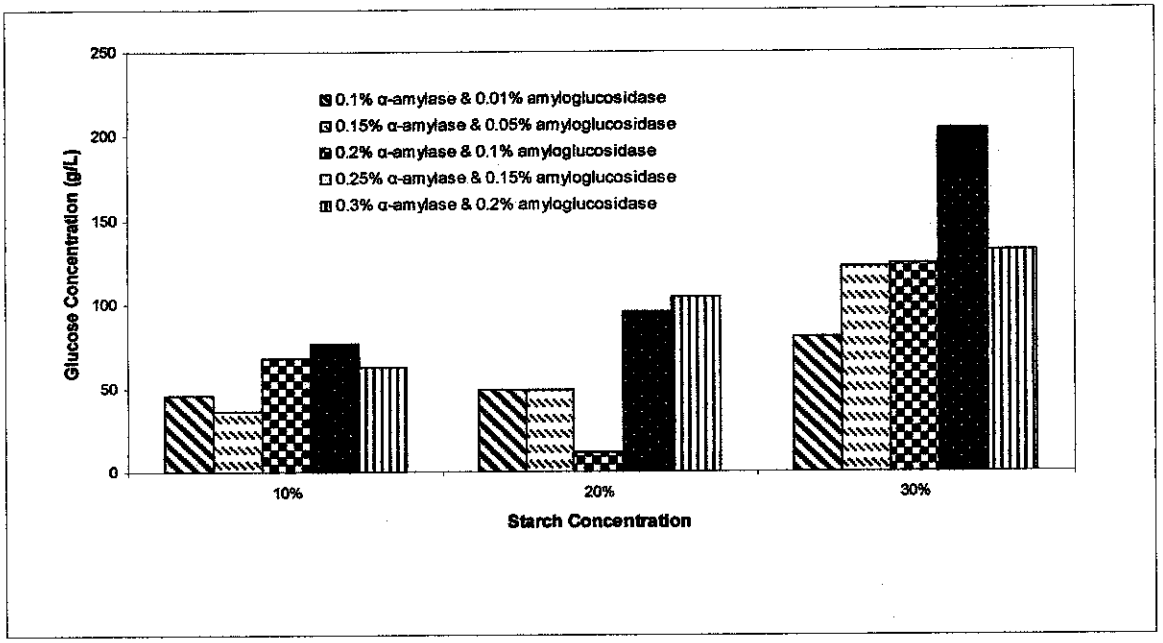


Figure 2: Effect of enzymes concentration on different starch concentration

The process of producing ethanol continues by converting sugar to ethanol by a reaction of yeast and sugar which is called as fermentation. Ethanol fermentation is the biological process by which sugars such as glucose, fructose, and sucrose are converted into cellular energy and thereby producing ethanol and carbon dioxide as metabolic waste products. Yeasts carry out ethanol fermentation on sugars in the absence of oxygen. Because the process does not require oxygen, ethanol fermentation is classified as anaerobic. Final product will contain ethanol and water. The pure ethanol can be obtained by the addition of molecular sieves or entrainer. Alcohol yields from raw cassava roots were between 82.3 and 99.6% (Seinosuke, 1980). The actual amount of cassava needed is dependent upon the starch content, but as a guide, cassava at 30% starch content will produce approximately 280 liters of alcohol/tonne. Cassava which is only 20% starch will produce only 180 liters of alcohol/tonne (cassavabiz, 2008).

Chemical reaction of fermentation of sugar by yeast:

$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2 + 2\ ATP$ (energy released:118 kJ/mol)

There are many types of yeast can be use in the fermentation process. The chosen of yeast is important to produce maximum amount of ethanol. Table 7 summarizes the ethanol production from 4 different types of yeasts.

Table 7: Ethanol production by monocultures of four yeasts using enzyme hydrolysed and unhydrolysed starch (Verma G. and Nigam P., 1999)

Cultures	Ethanol production				Fermentation efficiency (%)	
	S	AA-S	GA-S	AA+GA-S	S	AA-GA-S
S. diactaticus	16.8	32	24.8	34.4	38.1	78.1
E. capsularis	9.6	21.6	16.8	28	21.8	63.6
S. cerevisiae 21 (distiller's yeast)	-	-	37.6	37.6	-	85.4
S. cerevisiae BY (baker's yeast)	-	-	8	36	-	81.8

S= Unhydrolysed starch (control);
AA-S= α-amylase-treated starch;
GA= Glucoamylase treated starch;
AA+GA-S= α-amylase + glucoamylase treated starch

From the table, direct fermentation of starch to ethanol can be carried out effectively by using *S.cerevisiae* in the α-amylase and glucoamylase treated starch.

The amount of yeast is the other parameter affecting the rate of fermentation. 10% yeast inoculum may not sufficient to convert glucose to ethanol effectively (Ku Ismail, K. S, 2008). In figure 3, the rate of glucose consumption appeared to be slow and somehow became almost constant after 5 day. This condition is rather slow in terms of ethanol production capacity.

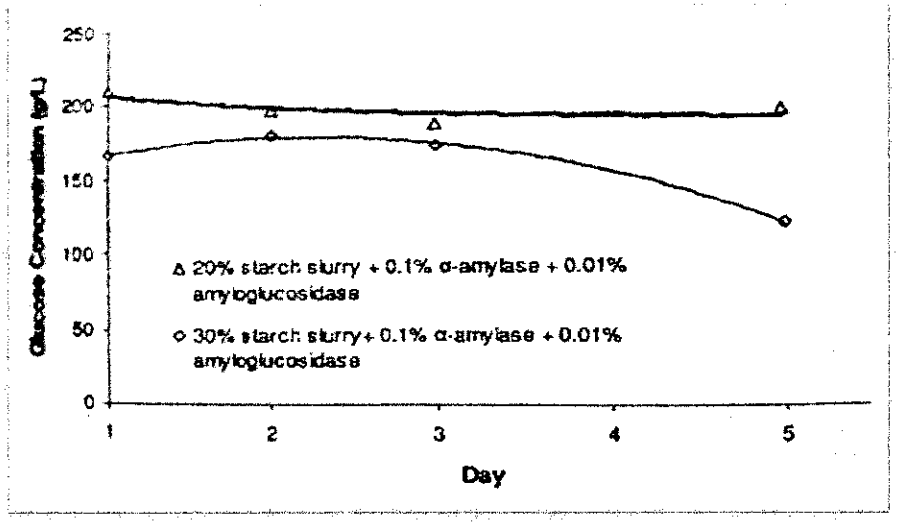


Figure 3: Reduction of glucose concentration during fermentation with 10% inoculum of *Saccharomyces cerevisiae*

2.6 Cassava waste

Production of cassava starch results in creation of around 10-15%of the original root weight as solid waste. This waste is known as 'pulp'. This pulp contain high starch content (50-60% dry basis), causing an environmental problem with disposal. Improved utilization of cassava waste will lead to efficient use of resource and less negative environmental impact. In order to recover this starch, physical or biological treatment of the material must be employed. Pulp is treated either by sonication or incubation with a multi-enzyme mixture of cellulase and pectinase. Starch recovery after physical disruption by sonication is approximately 15.69% of the pulp (dry weight basis). Treatment of pulp with cellulase and pectinase result in 40% (dry weight basis) starch recovery (Klanarong S. and Rungsima C, 1998). However, quality characteristics of liberated starch, including paste viscosity and thermal properties are comparable to a primary starch obtained by root extraction.

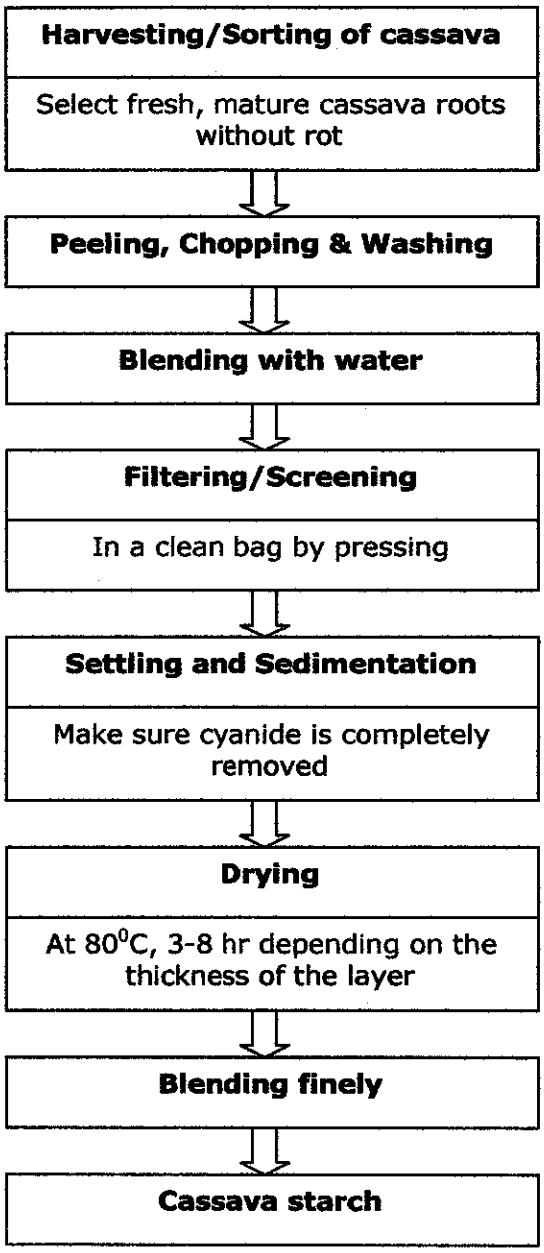
Table 8: Compositional analysis of cassava pulp

Composition	% (wet basis)	%(dry basis)
Starch	17.80 ±1.24	68.89 ± 4.00
Moisture	72.00 ± 0.08	-
Ash	0.44 ± 0.00	1.70 ± 0.01
Protein	0.4 ± 0.00	1.55 ± 0.03
Fiber	7.17 ± 0.06	27.75 ± 0.20
Fat	± ± 0.00	0.12 ± 0.01

CHAPTER 3

METHODOLOGY

3.1 Extraction of Cassava Starch



After selecting fresh and mature cassava, it is peeled and chopped into small cubes and soaked in water for 5 hours to remove the cynogenic content. The chips are washed and blended in water. The starch milk is obtained by straining the blends; a clean bag or filter is used by pressing, where the starchy water is separated from the fibre. Starch is obtained in sedimentation process for 3 to 5 hours. Two layers will form; the upper layer and bottom layer. The upper layer contains non-starchy water together with cyanide and should be discarded. The bottom layer is the starch sediment; white in color and fine texture. The process of sedimentation is repeated many times by removing and adding water until there is no more cyanide exists. Now, the starch sediment is dried in the oven at temperature of 70-80^{0}C for 3-8 hours depending on the thickness of the layer. The dried starch is then blended to obtain fine and smooth textures.

3.2 Gelatinization

Gelatinization is done by continuously heat the starch slurry above the heating plate until the structure become viscous and transparent. Upon continuous heating, the starch granules begin to disintegrate and the viscosity of the mixture begins to rapidly increase until it reaches a maximum where a paste is formed (Witt P. R. and Muscatine, 1983). Slurry of 20%, 30% and 40% starch is prepared.

3.3 Liquefaction

The heat stable alpha amylase is added to the each gelatinized starch at 80^{0}C with concentration of 0.1%, 0.15%, 0.2%, 0.25% and 30% (v/w). This enzyme, which is originated from *Bacillus amyloliquefaciens*, purchased from Sigma Alrich. One unit dextrinizes 5.46 dry starch per hour.

3.4 Saccharification

Once the liquefaction is done, different concentrations of amyloglucosidase; 0.01%, 0.05%, 0.1%, 0.15%, 0.2% are added into 5 samples of the liquefied starch (containing 0.1%, 0.15%, 0.2%, 0.25% and 0.3% α-amylase), respectively. Amyloglucosidase is produced by *Aspergillus niger*; one unit liberates 1 mg of glucose from starch in 3 minutes. The saccharification process is carried out at 60^0C for 24 hours in the water bath. The same procedure is repeated for 30% and 40% starch slurry. The glucose produced is analyzed using HPLC. A standard of glucose concentration is prepared by diluting 5 samples of 15, 20, 25, 30 and 40 mg/ml of D+. These samples are injected in HPLC in order to obtain a calibration curve for glucose.

3.5 Fermentation

Fermentation is done in the orbital shaker at 150 rpm with a temperature of 30^0C. In this process, the saccharified starch will react with 5 g of yeast (Saccharomyces cerevisiae) for 24-48 hours. The duration of fermentation is one of possible parameter that can be manipulated to produce maximum concentration of ethanol. The product of fermentation will be the mixture of ethanol, water and unfermented starch (residue). Ethanol can be obtained by distillation using simple distillation apparatus or rotary evaporator. The percentage of ethanol can be checked using Gas Chromatography (GC) or alcohol hydrometer.

3.6 Gantt Chart

No	Activities	5	6	7	8	9	10	11	12	13	14	15
1	Thermo-enzymatic hydrolysis											
2	Batch fermentation											
3	Filtration process											
4	Distillation of ethanol											
5	Redo the lab											
6	Progress report II											
7	Pre-EDX											
8	Final report											

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Preparation of cassava starch

It is observed that, 6 kg of unpeeled cassava is producing about 2 kg of cassava starch, about 33%, which is similar with study by Dai D. (2006). In order to improve the recovery of cassava starch, cassava pulp can be treated with with cellulase and pectinase result, which will result in 40% (dry weight basis) starch recovery (Klanarong S. and Rungsima C, 1998).

4.2 Gelatinization

From the conducted experiment, it is observed that as the starch undergoes continuous heating, the viscosity of the starch is kept increasing until a paste is formed. This paste is transparent with high viscosity and a bit sticky. This texture of gelatinized starch is similar as described by Witt P. R. and Muscatine, 1983. This is because, when an aqueous suspension of starch is heated, water molecules around the granule disrupt the hydrogen bonding and enter the granules which then swell. This process leads to the preparation of a viscous suspension. It is observed that 40% starch is the most viscous slurry, followed by 30% and 20% slurries. This is due to their starch concentrations. As the starch concentration increases, the ability of particles to move freely is decreases, and thus the viscosity is also increases.

4.3 Thermo-enzymatic hydrolysis

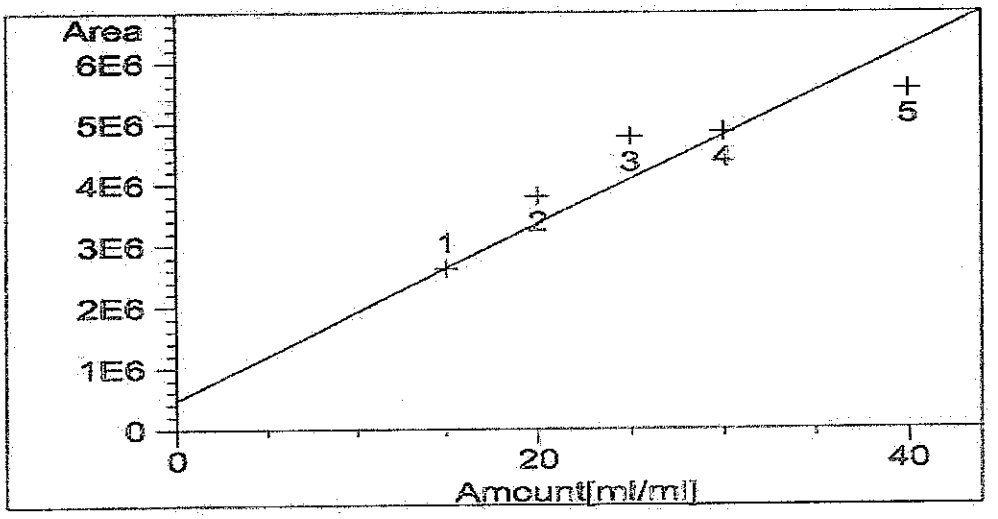


Figure 4: Calibration curve

Based on the calibration curve obtained above, it can be said that an error might have occurred during the process of preparing the standard, since not all the point is touched by the straight line and the correlation is only 0.96596. This will affect the results of glucose concentration.

By running all 15 samples of different enzyme concentrations with different enzyme concentrations using HPLC, the peak of refractive index signal for 30% starch slurry are indicates below:

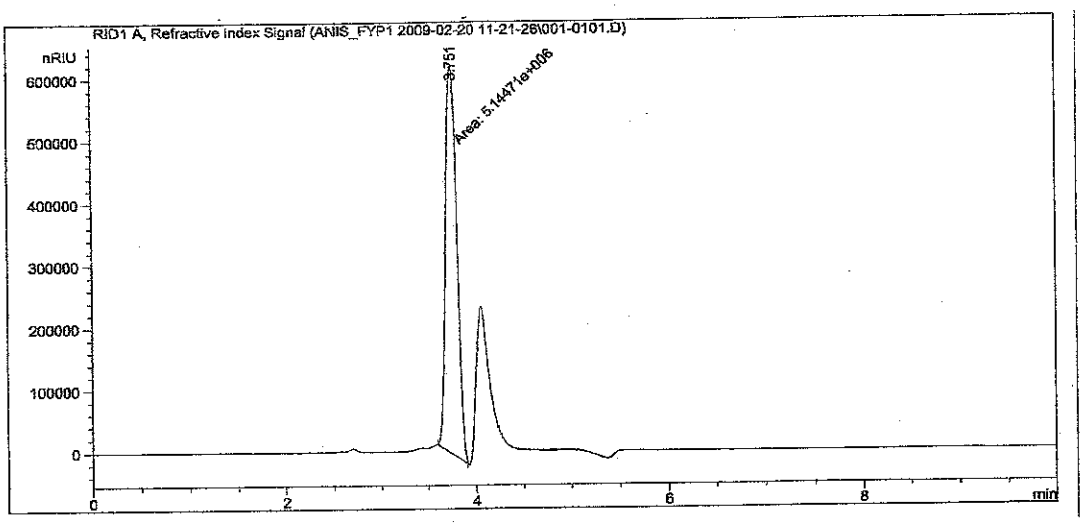


Figure 5: Peak area for slurry with 0.1% α-amylase + 0.01% amyloglucosidase for 30% starch slurry

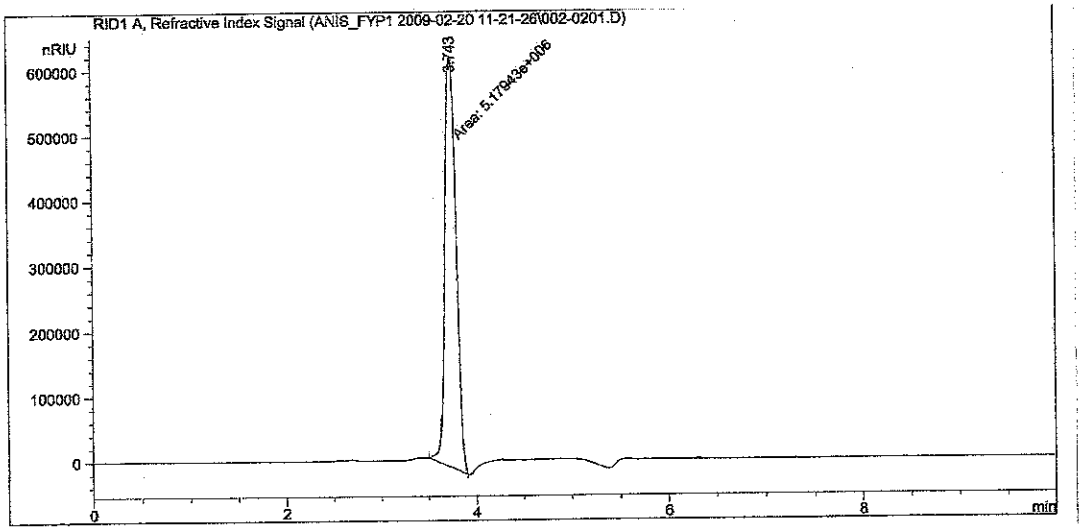


Figure 6: Peak area for slurry with 0.15% α-amylase + 0.05% amyloglucosidase for 30% starch slurry

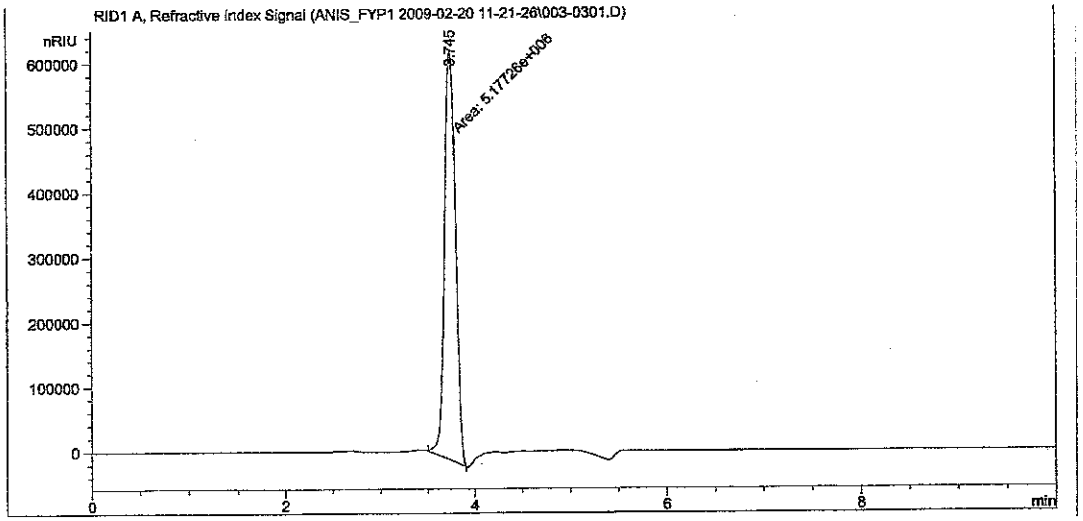


Figure 7: Peak area for slurry with 0.2% α-amylase + 0.1% amyloglucosidase for 30% starch slurry

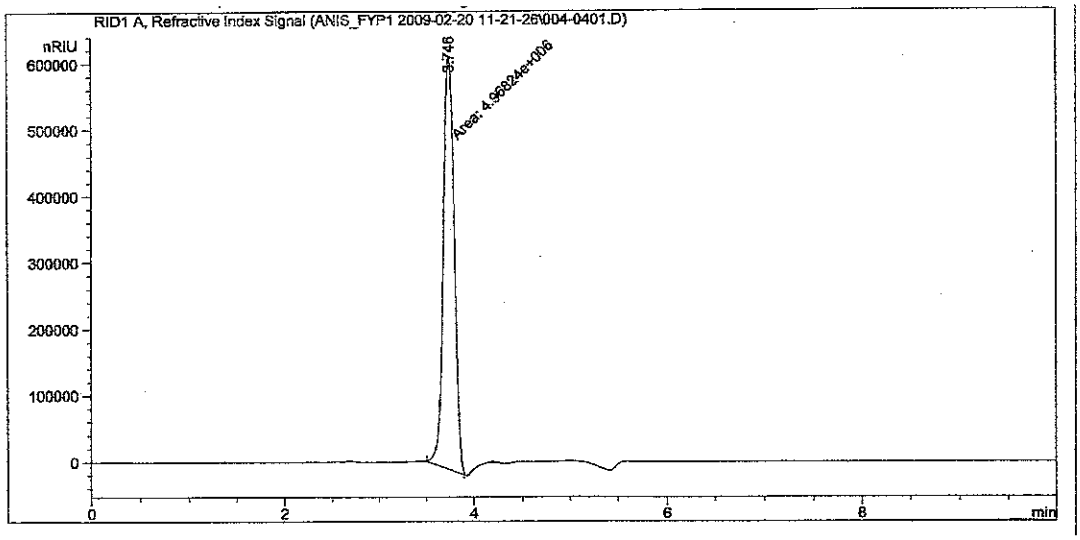


Figure 8: Peak area for slurry with 0.25% α-amylase + 0.15% amyloglucosidase for 30% starch slurry

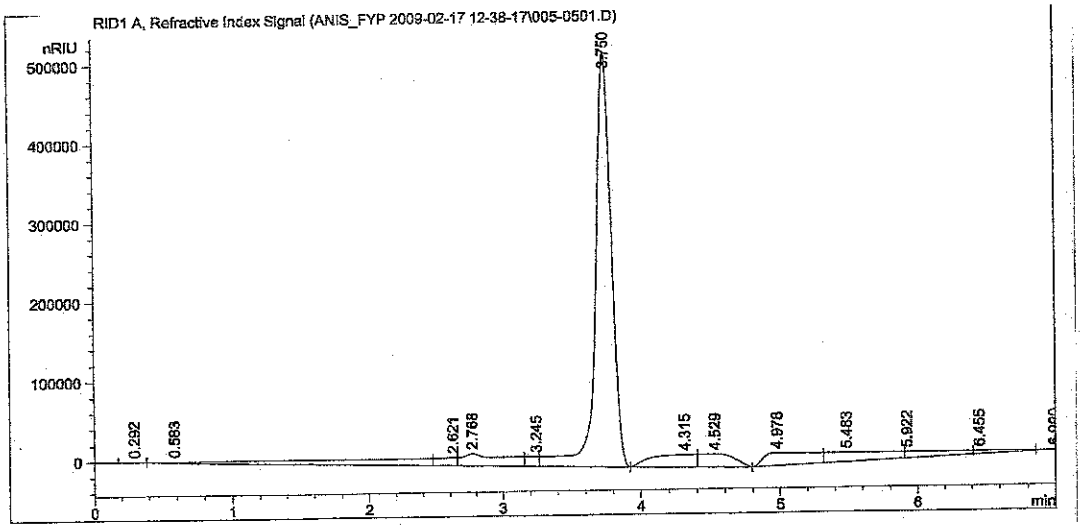


Figure 9: Peak area for slurry with 0.3% α-amylase + 0.2% amyloglucosidase for 30% starch slurry

The glucose is successfully detected around the retention time of 3.75 min. In order to know the amount of glucose in the samples, the area of each peak at 3.75 minutes is analyzed according to the calibration curve obtained before. Table 4 summarizes the concentration of glucose in each sample with different concentration of enzymes and starch:

Table 9: Glucose concentration with different concentration of enzymes in 20%, 30% and 40% starch slurry

SAMPLE		1	2	3	4	5
Starch slurry	Enzyme Concentration	0.1% α-amylase + 0.01% amyloglucosidase	0.15% α-amylase + 0.05% amyloglucosidase	0.2% α-amylase + 0.1% amyloglucosidase	0.25% α-amylase + 0.15% amyloglucosidase	0.3% α-amylase + 0.2% amyloglucosidase
20%	After dilution	25.86301	27.54601	25.71582	30.93665	27.37941
	Actual	232.7671	247.9141	231.4424	278.4299	246.4147
30%	After dilution	32.35815	32.59913	32.58409	35.57290	31.1334
	Actual	291.2234	293.3922	293.2568	320.1561	280.2003
40%	After dilution	36.12792	36.14562	37.89302	39.67821	39.23740
	Actual	325.1513	325.3106	341.0372	357.1039	353.1366

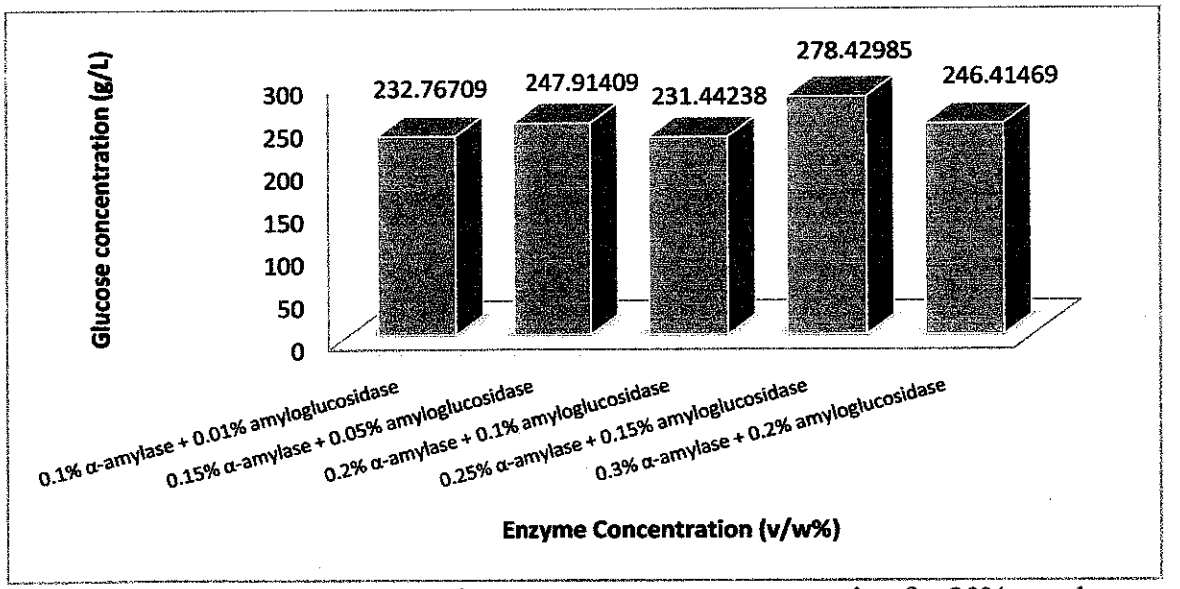


Figure 10: Glucose concentration versus enzymes concentration for 20% starch

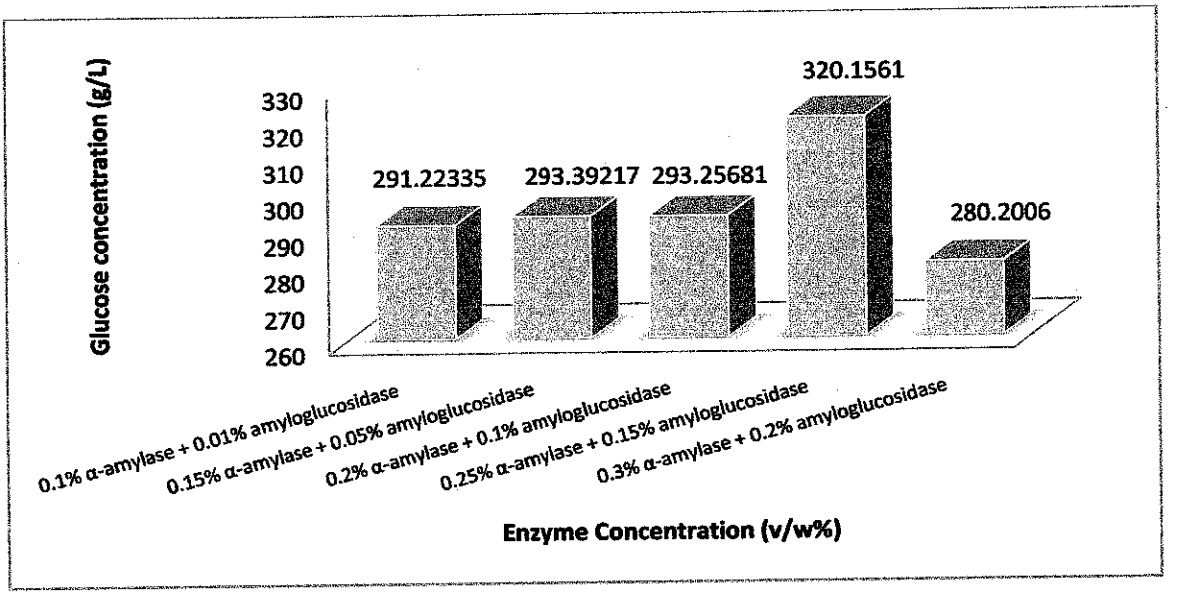


Figure 11: Glucose concentration versus enzymes concentration for 30% starch

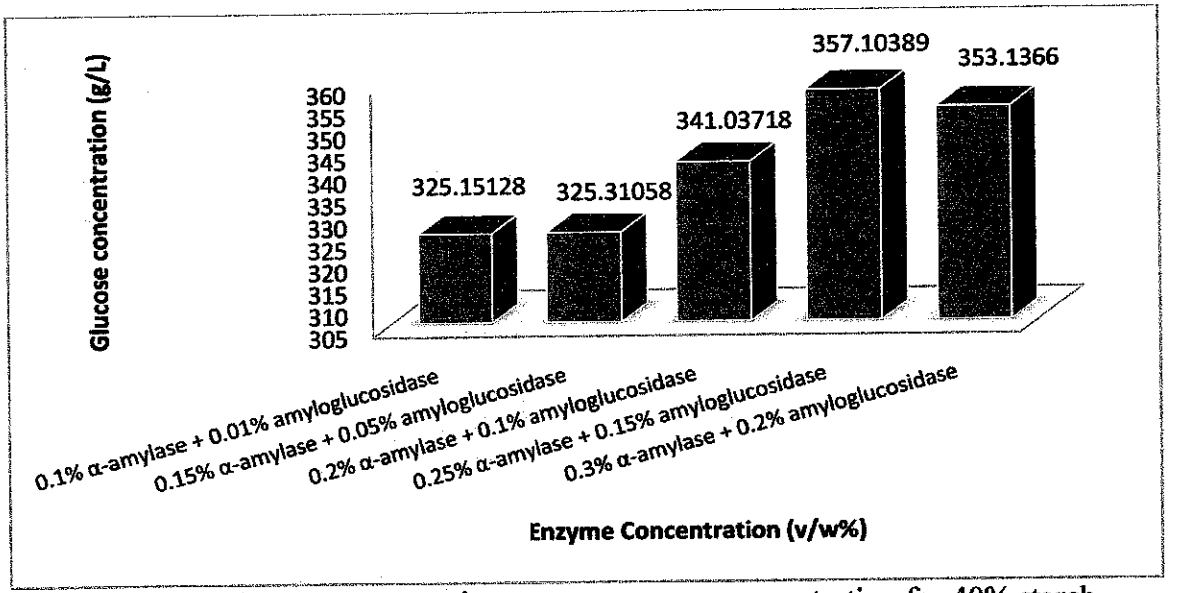


Figure 12: Glucose concentration versus enzymes concentration for 40% starch

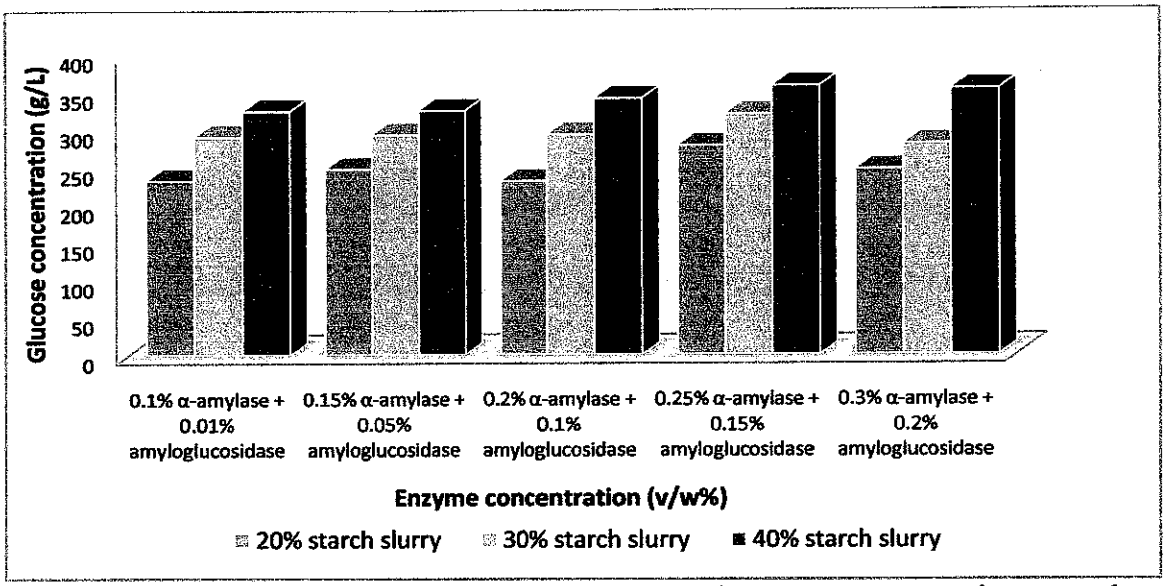


Figure 13: Effect of different starch concentration and enzyme concentration towards hydrolysis of cassava starch

The graph indicates that the maximum glucose concentration produced in the slurry with 0.25% α-amylase + 0.15% amyloglucosidase. This result is similar as indicated in Figure 2 by Ku Ismail K. S, 2008). However, the amount of glucose produced is much higher than her study since the author has saccharified the starch for 24 hours, which is the most effective saccharification period as reported by Aggarwal and Nigam, 2001.

However, the glucose concentration in each sample is found to be inconsistent in trend. The graph shows that at slurry of 20% and 30% with enzymes concentration of 0.15% α-amylase + 0.05% amyloglucosidase, the production of glucose is higher than sample 3, which consume greater amount of enzymes. Some errors might be committed during the experiment. One of the errors might be occurred during pipetting the enzyme using micropipette. Besides, the poor standard (calibration curve) might also contribute to this unrecognized trend.

4.4 Ethanol as a Product of Fermentation

Ethanol is obtained through distillation process at 78^0C. This temperature is an ethanol boiling point. Therefore, at this temperature, ethanol will evaporate and will then condense in the condenser and can be collected. However, in this experiment, there is no product. The ethanol is evaporate half way in the column and condensed. This might be due to length of fractional column. The ethanol evaporates and gets condensed by the ambient air before entering the condenser. Therefore, the rotary evaporator is used. However, there is still no product can be collected. The evaporated ethanol gets condensed in the round flask of the sample before being collected. Therefore, a higher temperature might have to increase so that the evaporated ethanol can enter the condenser and can be collected as product.

Since the author failed to collect the distillation product, the samples are directly taken to be analyzed in GC for its ethanol content. An ethanol standard is prepared and the following graph obtained:

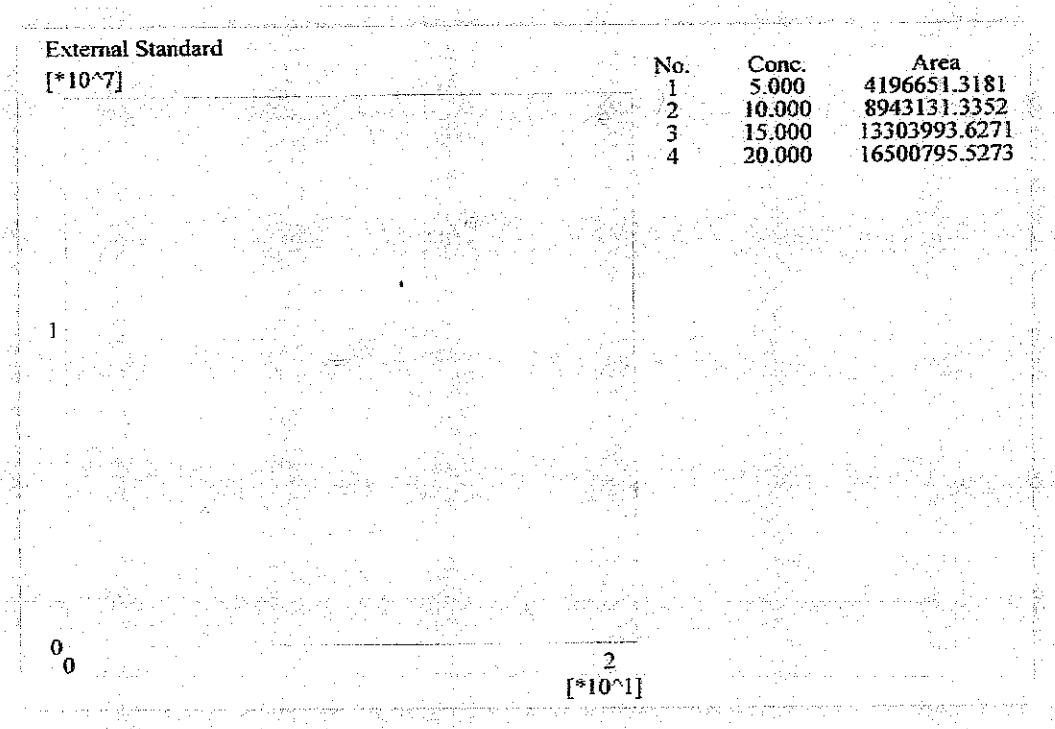


Figure 14: Calibration curve for ethanol

Ethanol is detected at retention time of 2.402 minutes. Table 10 summarizes the percentages of ethanol obtained from different concentration of glucose and starch.

Table 10: Percentage of ethanol in fermented starch

SAMPLE	1	2	3	4	5
Enzyme Concen-tration / Starch slurry	0.1% α-amylase + 0.01% amyloglucosidase	0.15% α-amylase + 0.05% amyloglucosidase	0.2% α-amylase + 0.1% amyloglucosidase	0.25% α-amylase + 0.15% amyloglucosidase	0.3% α-amylase + 0.2% amyloglucosidase
20%	11.5215%	11.7564%	11.5177%	13.8651%	11.6394%
30%	14.1865%	14.5643%	14.2536%	15.9727%	14.0856%
40%	16.7856%	16.7146%	17.864%	19.8213%	19.3817%

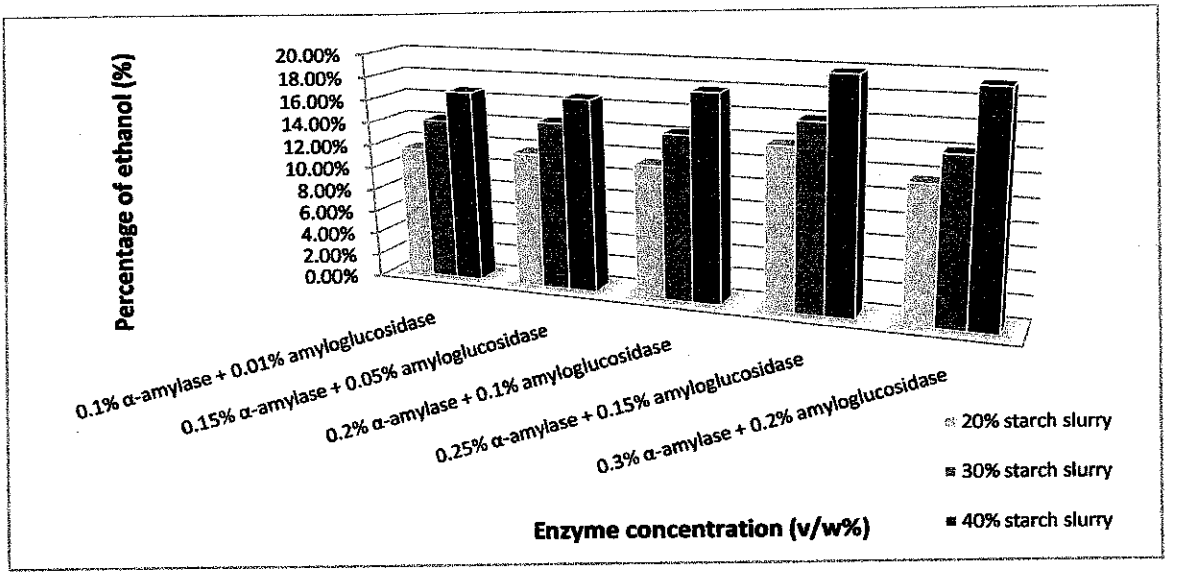


Figure 15: Percentage of ethanol vs enzyme concentration

As shown in the figure above, optimum enzyme for 20% slurry (0.25% α-amylase + 0.15% amyloglucosidase) yield about 14% ethanol. For 30% slurry with optimum enzyme concentration, 16% of ethanol is obtained and for 40% slurry with same amount of enzyme, 20% of ethanol is produced. From this percentage, the rate of ethanol obtained per amount of cassava starch is known. For 20% starch, with 14% ethanol from 100 ml fermented starch, the amount of ethanol is 14 ml from 20 g starch or 0.7 liter per kg of cassava starch. For 30% starch, with 16% ethanol from 100 ml fermented starch, the amount of ethanol is 16 ml from 30 g starch or 0.53 liter per kg of cassava starch. For 40% starch, with 20% ethanol from 100 ml fermented starch, the amount of ethanol is 40 ml from 40 g starch or 0.5 liter per kg of cassava starch. Comparing these results with Seinosuke studies, who produce around 82.3 and 99.6%, these amounts of ethanol are considered low in term of ethanol production. It can be said that 5 gram of yeast is not sufficient for ethanol production. However, these results still can be accepted since the other parameters such as environment condition and optimum pH of hydrolysis are not taken in to account. If this experiment is conducted in sterilized condition with an optimum pH, the production of ethanol can be improved. As a conclusion, in order to

improve the yield of ethanol, the production of glucose should be optimized first by considering all parameters and once the glucose production is optimized, amount of yeast for fermentation should be manipulated and supplement for yeast should be added.

CONCLUSIONS AND RECOMMENDATIONS

The objective of this project is achieved by producing ethanol bio-fuel from bitter type of cassava. The effect of starch and enzymes concentration towards the glucose production has been studied and the texture of starch during the gelatinization process has been observed; the fermentation process of ethanol from Saccharomyces cerevisiae has been successfully carried out, and the rate of ethanol production has been calculated by obtaining amount of glucose produced per 1 kg of cassava starch.

From the literature, there are many factors can be manipulated in order to produce the maximum amount of ethanol. The optimum enzyme concentration for liquefaction is around 0.25%(v/w) and 0.15%(v/w) for saccharification using amyloglucosidase. The optimum pH for both liquefaction and saccharification is found to be 5.0. Since in literature, 10% of yeast is considered insufficient to produce effective amount of ethanol, the author suggests to increase the amount of yeast to 30%.

The starch from cassava pulp can be recovered through a treatment with cellulose and pectinase so that more starch can be produced from cassava. In order to improve the amount of ethanol obtained, a lot of parameter should be considered. This experiment should be conducted in sterilized condition. All the equipments should be heated in high pressure and temperature using autoclave, the experiment should be conducted in laminar flow cabinet, and the author's hand should be cleaned by splattering 10% ethanol. Besides, thermo-enzymatic hydrolysis should be conducted in its optimum pH (5.0) and the production of glucose should be optimized as well as the amount of yeast used. The dose of α-amylase used in this experiment can be reduced by the addition of certain amount calcium chloride ($CaCl_2$). Other than the parameter and condition explained above, the yeast should be supplied with nutrient by preparing a broth culture of yeast containing peptone, yeast extract and glucose. For the further purpose of

optimization, software called Design Expert can be used since there are many parameters in this experiment.

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