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Bio-Solvent Preparation from Apple Biomass for Pharmaceutical, Cosmetic and Biomedical Industrial Application

A.B.M. Sharif Hossain
Programme of Biotechnology, Department of Biology, Faculty of Science, University of Hail, Hail 2440, KSA.

Introduction

Bioethanol can be used as biosolvent in the laboratory, pharmaceutical, cosmetic, medical and biomedical industries. It is generated by the means of utilizing biological materials such as trees, woods, fruits, algae or biomass to make solvent in the laborator and generate electricity. Biomass is biodegradable, sustainable, economic sources as well as renewable and biomaterial resource for the manufacturing industries like bio-solvent, bio-plastics, bio-film, biomaterials, bio-chemicals, bio-fuels, bio-electricity, in the agro-industry, pharmaceuticals, biomedical and bioengineering (Hossain et al, 2008). The importance of biomaterial, fermenter and solvent are increasing exponentially to meet the demands of industrialization and population explosion worldwide since last couple of decades (Hossain et al, 2008). The use of this agricultural derived, apple waste which can be a technique of clean technology like environment pollution, fossil-fuel depletion and waste management. It can be a viable alternative, a biosolvent should provide a net energy gain, have environmental benefits, be economically competitive, and be producible in large quantities without reducing food supplies (Hill *et al*., 2006). Bioethanol is an alkyl-alcohol produced from fermentation of biological matters that possess sugar, starch or cellulose. Alcoholic fermentation is the conversion of sugar to carbon dioxide and ethanol (ethyl alcohol) by using zymase from yeast. Fermentation using genetically engineered yeast or bacteria will utilize all five of the major biomass sugars: glucose, xylose, mannose, galactose and arabinose. Bioethanol may be produced by direct fermentation of sugars, or from other carbohydrates that can be converted to sugar, such as starch and cellulose (Demirbas, 2005). Enzymatic hydrolysis requires feedstock pretreatment, enzyme production, and enzyme recovery, which may make this option economically unfeasible (Iranmahboob *et al*., 2001). The chemical reaction is shown below:

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + \underset{\text{Water}}{H_2O} \xrightarrow[\text{Catalyst}]{\text{Invertase}} \underset{\text{Fructose}}{C_6H_{12}O_6} + \underset{\text{Glucose}}{C_6H_{12}O_6}$$

$$\underset{\text{Fructose / Glucose}}{C_6H_{12}O_6} \xrightarrow[\text{Catalyst}]{\text{Zymase}} \underset{\text{Ethanol}}{2C_2H_5OH} + 2CO_2$$

Bioethanol has been made using apple (Chatanta *et al*., 2008 and), date, grape (Najafi *et al*., 2008), mango, pineapple, banana and cashew apple (Rocha *et al*., 2008, Hossain et al, 2011). Bioethanol can be produced from fruit residues like pineapple, banana peel, apple and mango fruit residues and other sources like algal biomass by the use of yeast, cellulase, lipase and amylase fermentation bioprocess technology [Hossain et al, 2008], [Hossain et al, 2007]. It is reported that the pharmaceutical industry applies ethanol in its purest profile into the production of drugs and homoeopathic products, disinfectants, as well as extraction of plant agents. Equally the cosmetic industry works with sensory neutral ethanol for the production of perfumes, as well as neutral carrier substance for fragrances. Ethanol is also traceable in cosmetics as ingredient, solvent, and also as neutral preservative agent (BAH, 2014. Ethimex (2014) stated anhydrous Ethanol is produced via fermentation and dehydration. With a water content of less than 0.1%, Absolute Alcohol is widely used in pharmaceuticals, cosmetics and inks, as well as in certain fine chemical processes. Extra-Neutral Alcohol (ENA), also known as Extra-Fine Alcohol or Surfin, is a high-purity ethyl alcohol generally produced from sugarcane molasses or grain via fermentation, distillation and precision rectification. It is widely used in pharmaceutical, perfumery and cosmetics applications, as well as premium beverage (especially premium vodkas, premium white spirits, and aperitifs) and fine chemical formulations. Few literatures are found in this regard. Therefore the objectives of the study were undertaken to optimize the bioethanol production from waste apple and to evaluate the properties and quality of bioethanol produced from apple biomass.

Materials and Methods

Materials

The main ingredient was rotten Fuji Apple, bought from a fruit shop at Pantai Dalam, Kuala Lumpur. After collection, samples were kept for one week until it rots evenly.

Micro-organism

Saccharomyces cerevisiae Type II (Baker's Yeast) supplied from ABO Laboratory. *S. cerevisiae* was rehydrated to activate it by heating for 15 minutes at 40°C water bath after adding 10% water.

Enzymes

Enzymes used were cellulase from *Aspergillus niger* and α-amylase from *Bacillus* species. Both were supplied from ABO Laboratory.

Chemical and Reagents

Chemicals used were NaOH, HCl, potassium dichromate, s-Dipehenylcarbazide, dinitrosalicylic acid, phenol, sodium sulfite and sodium hydroxide and Rochelle salt. All were supplied by Chemo lab.

Methods

Sample collection and processing

To obtain sample mixture, Wareing Blender was used. Samples were blended for about 20 minutes. When juice and pomace were used, samples must be blended with Philips Juicer 550w. Direct separation of juice and pomace were obtained at short interval. pH and Total Soluble Solid (TSS) of samples were accessed using pH meter and refractometer.

Fermentation

Water was added with 10% for juice, 30% for mixture and 50% for pomace. Then 100 ml of samples were put in 500ml Schott Bottles in triplicates. 2g/l of dry *Saccharomyces cerevisiae* Type II supplied from ABO lab was weighed and then rehydrated. Yeast and samples were mixed inside Schott Bottles to ensure efficient fermentation. Samples were labeled properly, capped tightly and stored in incubator at 32°C for two days.

Temperature Parameter

The process was the same as mentioned in Fig. 1 but temperature for incubation was varied to 28°C, 32°C and 36°C.

Fermentation of Parameter of pH

The process was the same as mentioned in Fig. 1 but pH of samples before incubation were varied to pH 4, 5 and 6 by adding NaOH to increase pH or HCl to reduce pH.

Fermentation of Fruit Part

Apples were process by Wareing Blender to give mixture while Philips Juicer to give juice and pomace. Then process as mentioned in Fig. 1 was conducted.

Fermentation of Fruit Condition

Fresh and rotten apple were blended separately to obtain mixture. Then process as mentioned in Fig. 1 was conducted.

Enzymatic Hydrolysis of Apple Mixture

Enzymatic hydrolysis using cellulase and amylase were done. 3mg/l of each enzyme were weighed and add with 2g/l yeast. Then process in Fig. 1 was conducted.

Filtration

After two days, fermentation broth was filtered through two layers of cheese cloth. Allow the apparatus to settle down for one hour to ensure all liquid had drained out from residue. Volume of liquid which was the raw bioethanol and weight of residue were taken. Raw bioethanol was then purified by vaccum evaporator in the laboratory. TSS and pH of bioethanol were measured.

Sample Analysis

Ethanol Assay

Ethanol assay were done using Dichromate Colorimetric Method by Williams and Reese (1950). Ethanol standard curve was plotted by taking absorbances of known ethanol concentrations. 1 ml of ethanol solution was pipette into glucose sample in a lightly capped test tube. Test tube was covered with parafilm to avoid loss of liquid by evaporation. Then the mixture was heated to at 90°C for 5 to 15 minutes to develop red-brown color. 1ml of Rochelle salt was added to stabilize the color. The absorbances were taken at 575nm after cooling the samples at room temperature. Then absorbances were compared with ethanol standard curve and ethanol concentration of the sample was read.

Glucose Assay

Measured using Dinitrosalicylic Colorimetric Method by Miller, (1959). A standard was prepared by measuring absorbances of diluted 0.5mg/ml glucose solution. 3ml of DNS reagent was added to 3ml glucose sample in a lightly capped test tube and cover with parafilm. Then the mixture was heated to at 90°C for 5 to 15 minutes to develop red-brown color. 1ml of Rochelle salt was added to stabilize the color. The absorbances were taken at 575nm after cooling the samples at room temperature. Absorbances were compared with standard curve and the glucose concentrations were read.

Chemical Analysis

Samples of bioethanol fermented at 28°C, 32°C and 36°C were sent to Tribology Laboratory at Faculty of Engineering, UM. By using multi element oil analyzer (MOA II), lube oil analysis was conducted to all three samples to determine amount of chemical and metal inside bioethanol.

Methodology

Figure 1a: Processing and preparing rotten apple for fermentation

Figure 1b: Fermentation of rotten Fuji Apples.

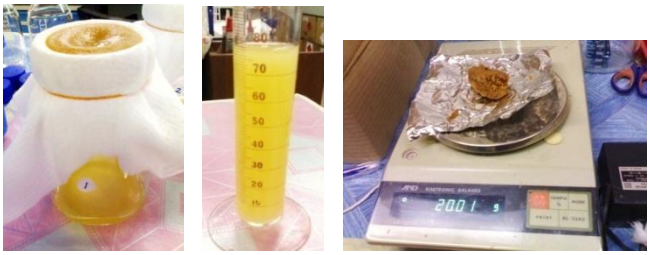

Figure 1c: Filtration of liquid and residue after two days

Figure 1d: pH determination from bioethanol for (left), glucose assay (middle)

Results and Discussion

Optimizing parameters for bioethanol production

Effect of temperature on bioethanol production

Fermentation of rotten Fuji apples was conducted at 28 °C, 32 °C and 36 °C for two days. Then sample filtration and Dichromate Colorimetric Method was done to measure bioethanol yield.

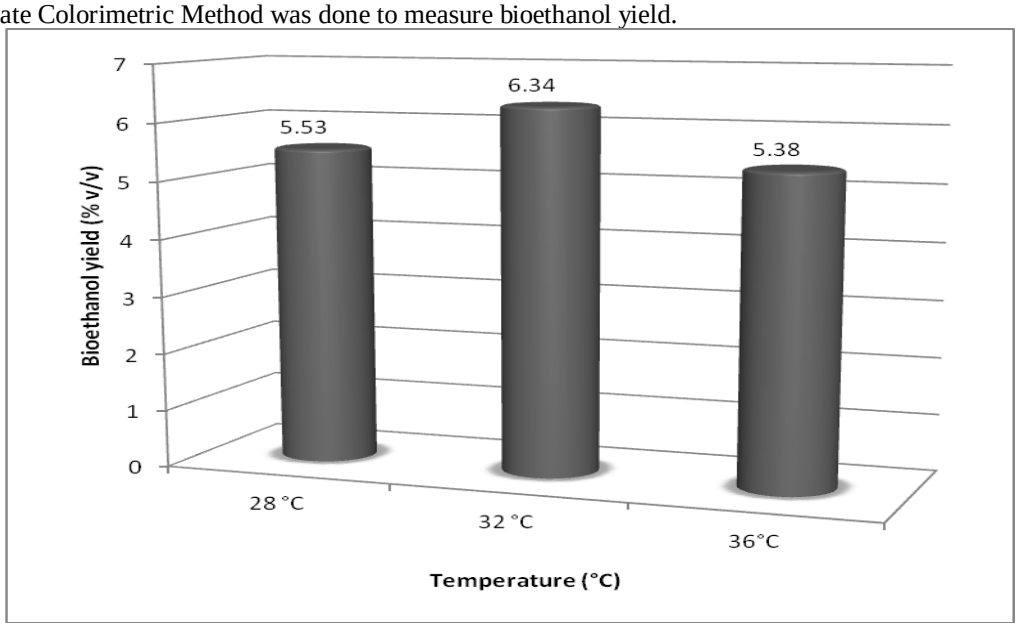

Figure 2: Effect of temperature on bioethanol production

From Figure 2, the highest bioethanol yield was 6.34% (v/v) at 32°C, followed by 5.53% (v/v) at 28°C and the least was at 36°C, where bioethanol yield was 5.38% (v/v). ANOVA test was conducted and p was less than 0.05. This showed result was significant.

Effect of pH on bioethanol production

Fermentation was done by fixing apple mixture pH to pH 4, 5 and 6 to find the best pH for bioethanol production.

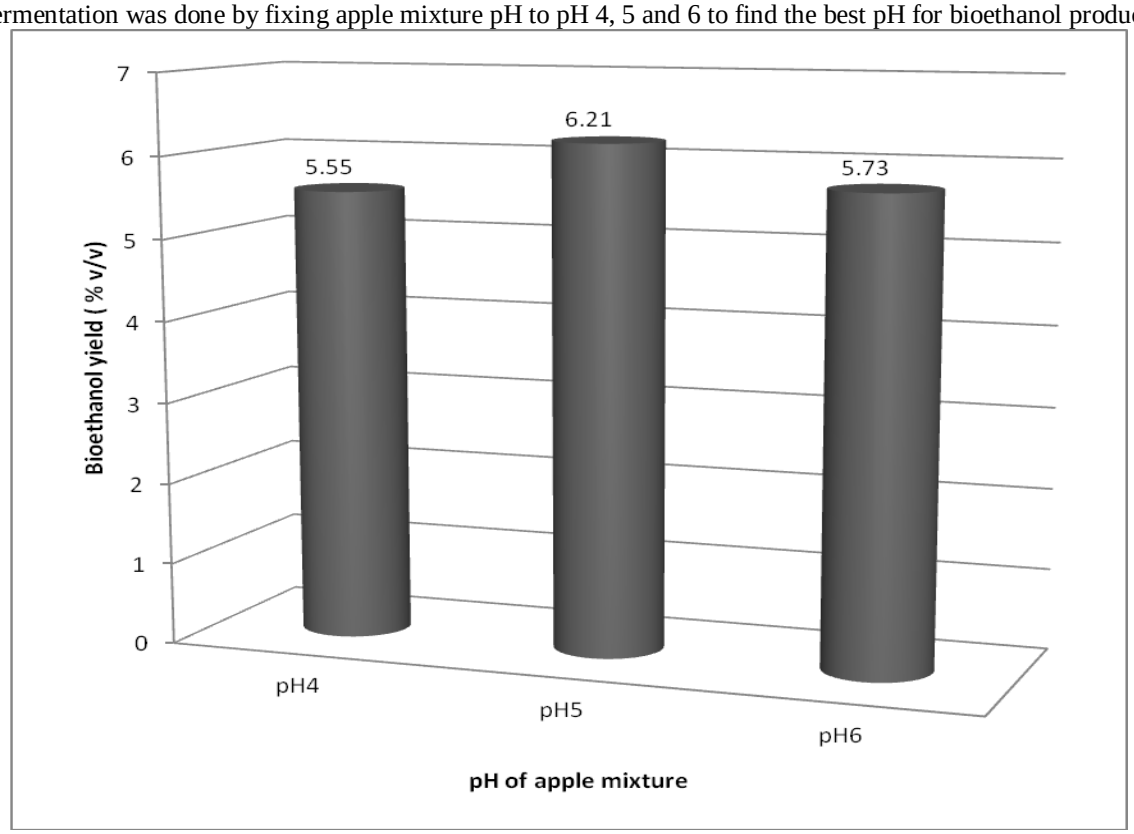

Figure 13: Effect of pH on bioethanol production

As in Figure 3, the highest bioethanol yield was at pH 5, followed by pH 6 and the least at pH 4 with bioethanol yield of 6.21, 5.73 and 5.54% (v/v) respectively. ANOVA test was conducted and p was less than 0.05. This showed result was significant.

Effect of fruit part on bioethanol production

Juice, mixture and pomace of rotten Fuji apple were fermented with 2g/l *S. cerevisiae*. After two days, bioethanol yield were measured.

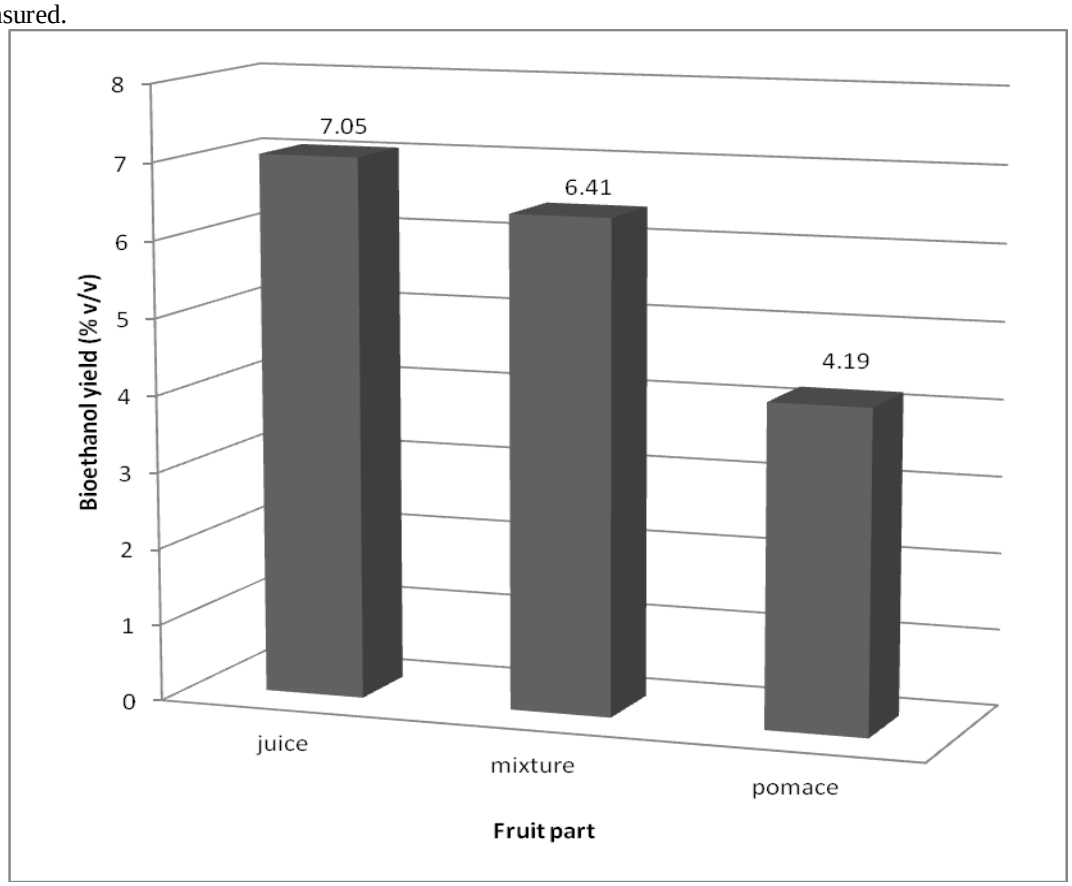

Figure 4: Effect of fruit part on bioethanol production

From the experiment, Fuji apple juice had highest bioethanol yield when fermented for two days, followed by mixture and pomace. In Figure 4, bioethanol yields were 7.05% (v/v) for juice, 6.41% (v/v) for mixture and 4.19% (v/v) when pomace was used as substrate for solid state fermentation. ANOVA test was conducted and p was less than 0.05. This showed result was significant.

Effect of fruit condition on bioethanol production

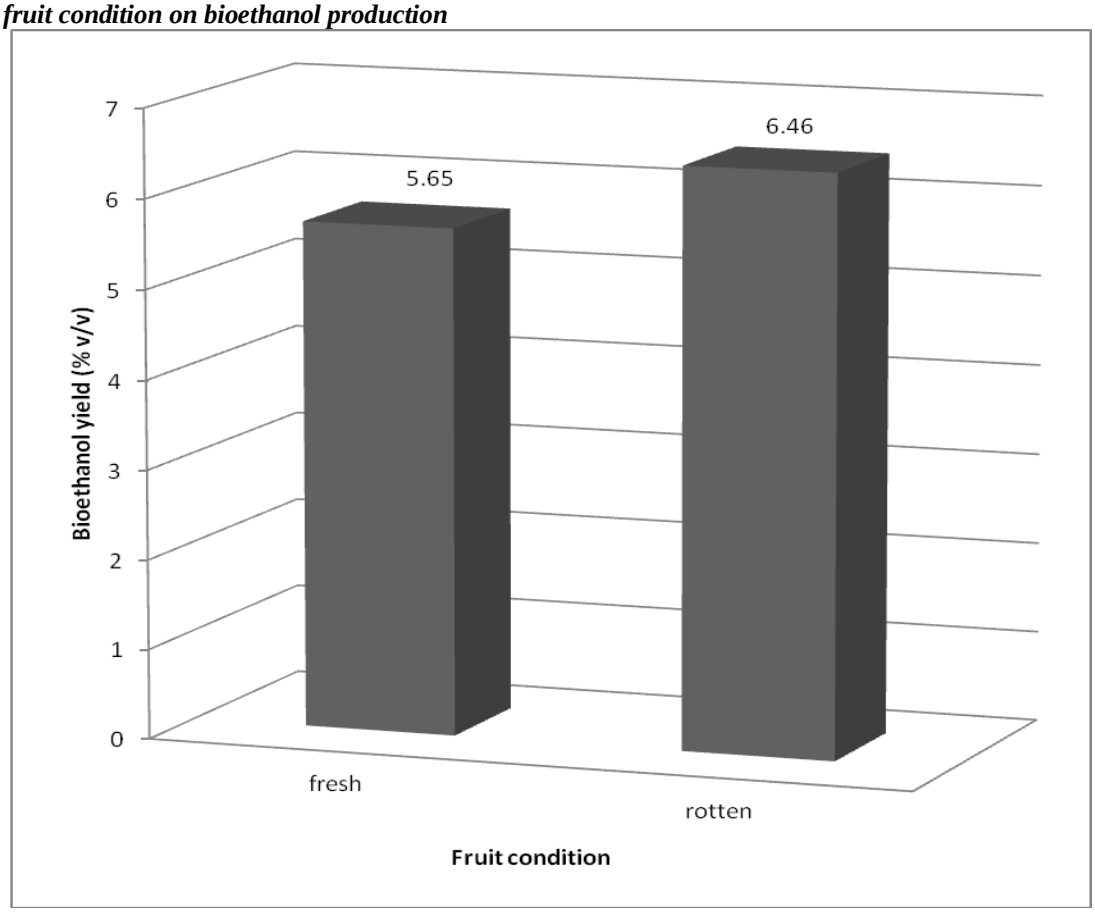

Figure 5: Effect of fruit condition on bioethanol production

As in Figure 5, the highest yield was by fermentation on rotten apple, 6.46% (v/v). bioethanol from fresh apple was lower, 5.64% (v/v). ANOVA test was conducted and p was less than 0.05. This showed result was significant.

Effect of enzymatic hydrolysis on bioethanol production

Three parameters were tested; fermentation using yeast, yeast mixed with α-amylase and yeast mixed with cellulase.

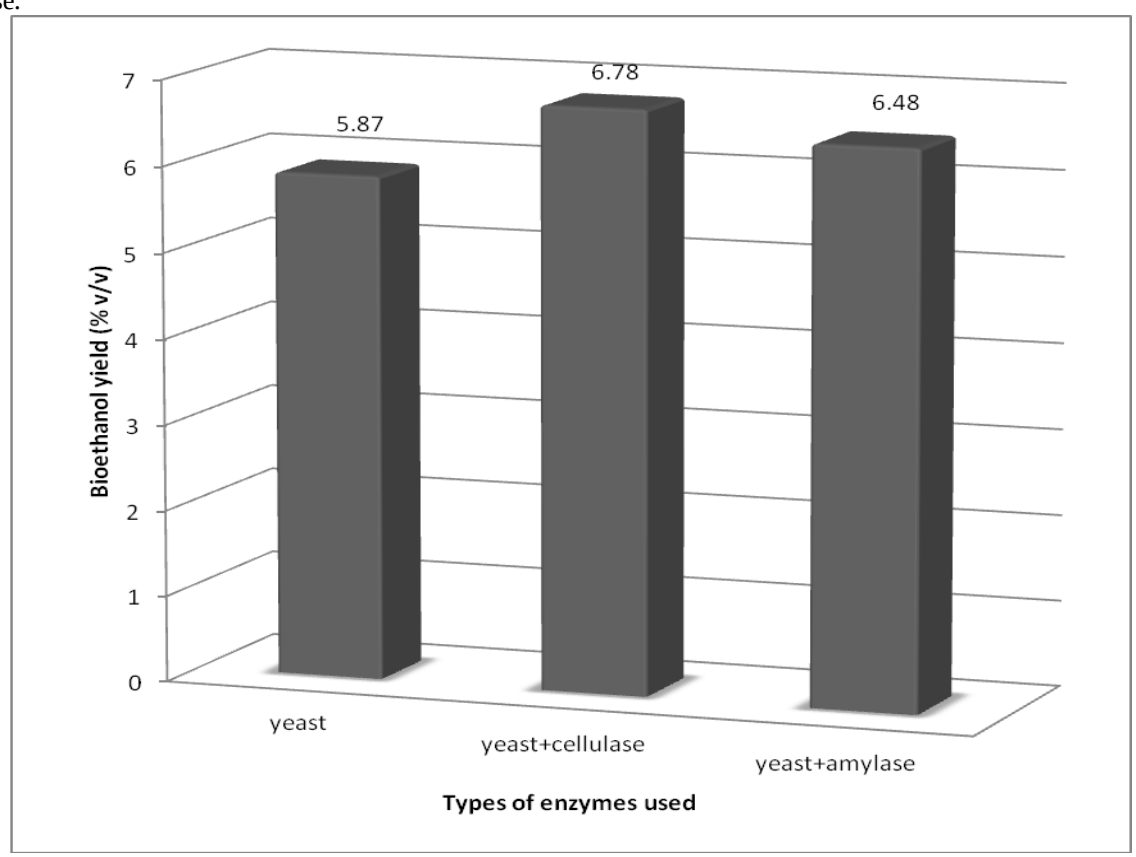

Figure 6: Effect of enzymatic hydrolysis on bioethanol production

From Figure 6, it was clearly shown that fermentation using yeast and cellulase mixture yield the most bioethanol, 6.78% (v/v), the second highest was mixture of yeast and α-amylase, 6.48% (v/v) and the lowest bioethanol yield was 5.87% (v/v) when yeast alone was used for two days fermentation. ANOVA test was conducted and p was less than 0.05. This showed result was significant.

Analysis of bioethanol

pH before and after fermentation

The initial pH was set to 5.0 and fermentations were done at 28°C, 32°C and 36 °C. After two days, bioethanol yields were analyzed.

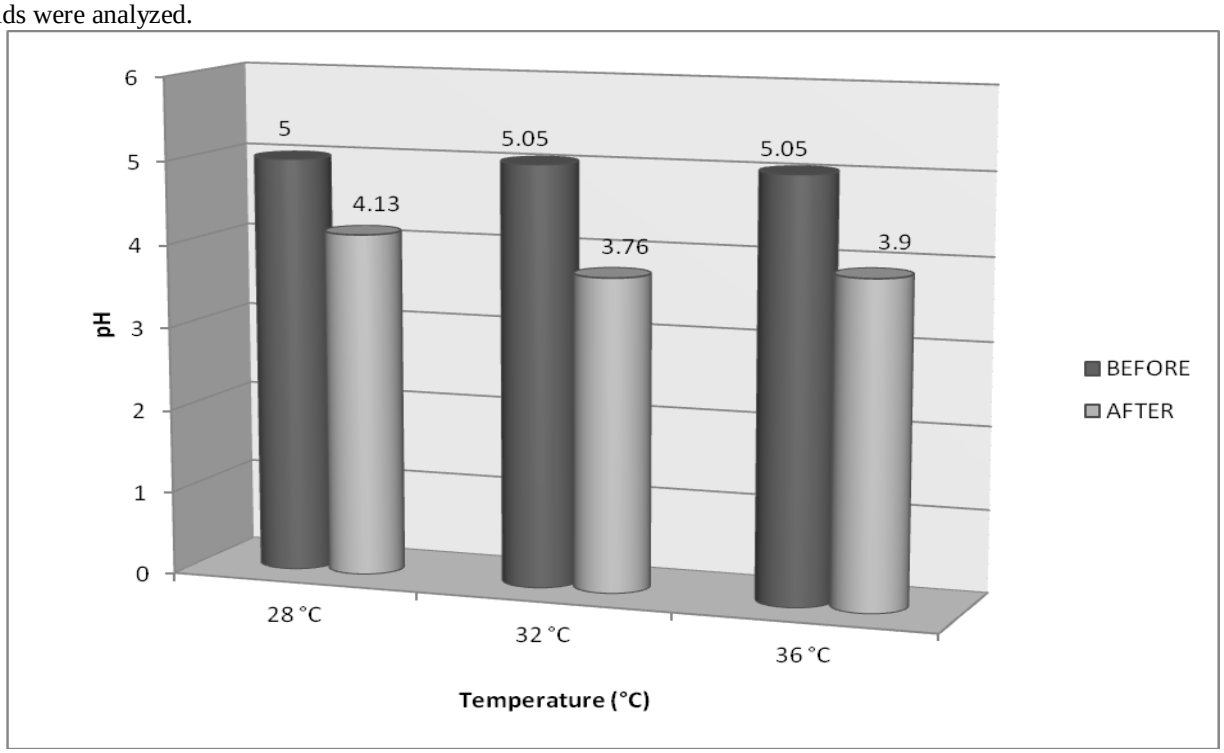

Figure 7: pH before and after fermentation

Figure 7 showed the differences before and after fermentation, with temperature 32°C having the least pH after fermentation (pH 3.76), followed by 36°C (pH 3.7) and the highest pH after fermentation was reported at 28 °C (pH 4.13). ANOVA test was conducted and p was less than 0.05. This showed result was significant.

TSS before and after fermentation

The initial TSS of rotten apple mixture was measured by refractometer to be 10.8°Brix. After fermentation, bioethanol TSS were measured.

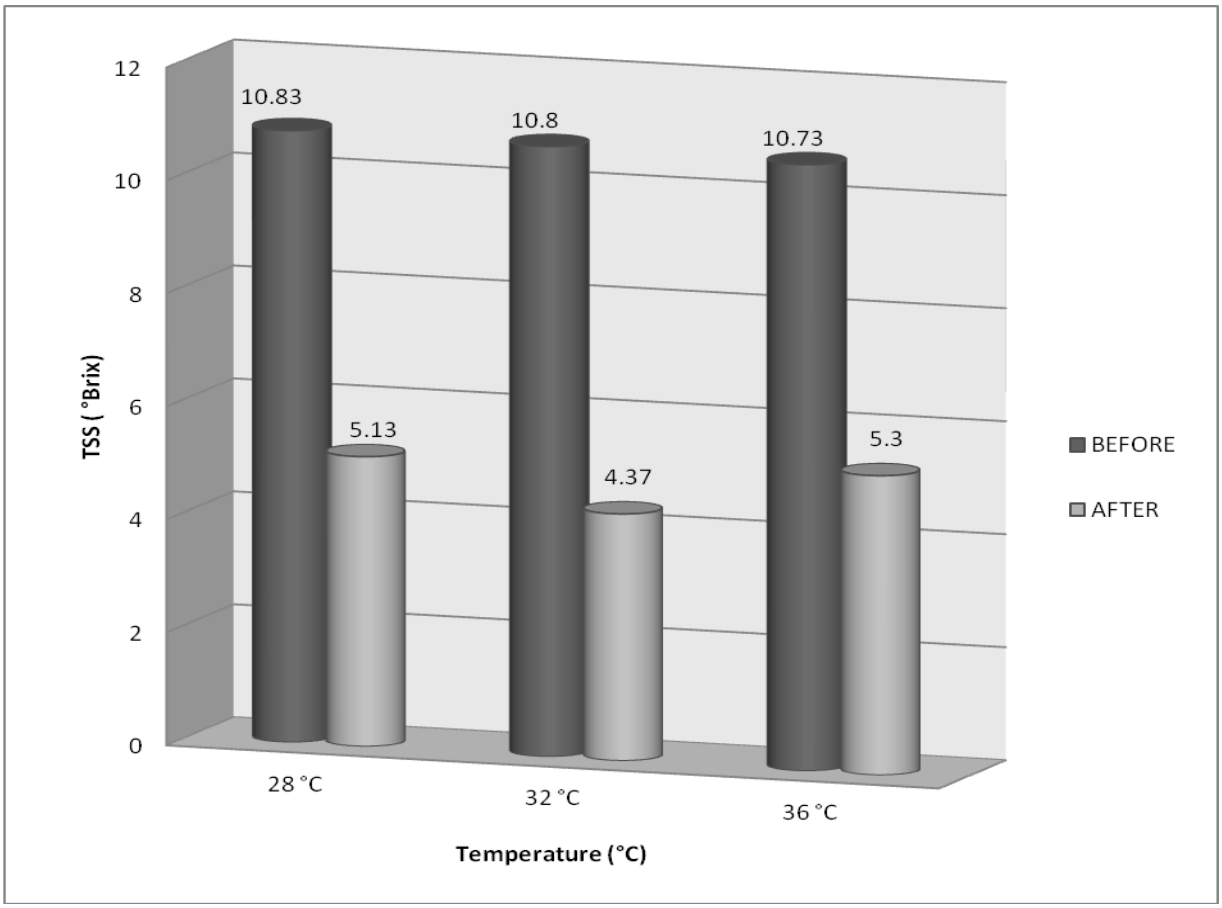

Figure 8: TSS before and after fermentation.

From Figure 8, the lowest TSS after fermentation was at 32 °C which was 4.37°Brix followed by 5.13°Brix at 28 °C and the highest TSS after fermentation was at 36°C, 5.3° Brix. ANOVA test was conducted and p was less than 0.05. This showed result was significant.

Chemical analysis

Samples of bioethanol produced by fermentation at 28 °C, 32 °C and 36 °C were analyzed for chemical and metal components.

Table 1. Chemical component determination

Temperature	Na	P	Ca	Mg
28 ^{0}C	0.3	5	2.5	3.4
30 ^{0}C	0.2	2.5	1.5	2.2
36 ^{0}C	0.3	3.5	2.7	2.1

Table 1, shows the standard value of chemical components of Na, Ca, P, Mg whict might come from the samples.

Glucose analysis

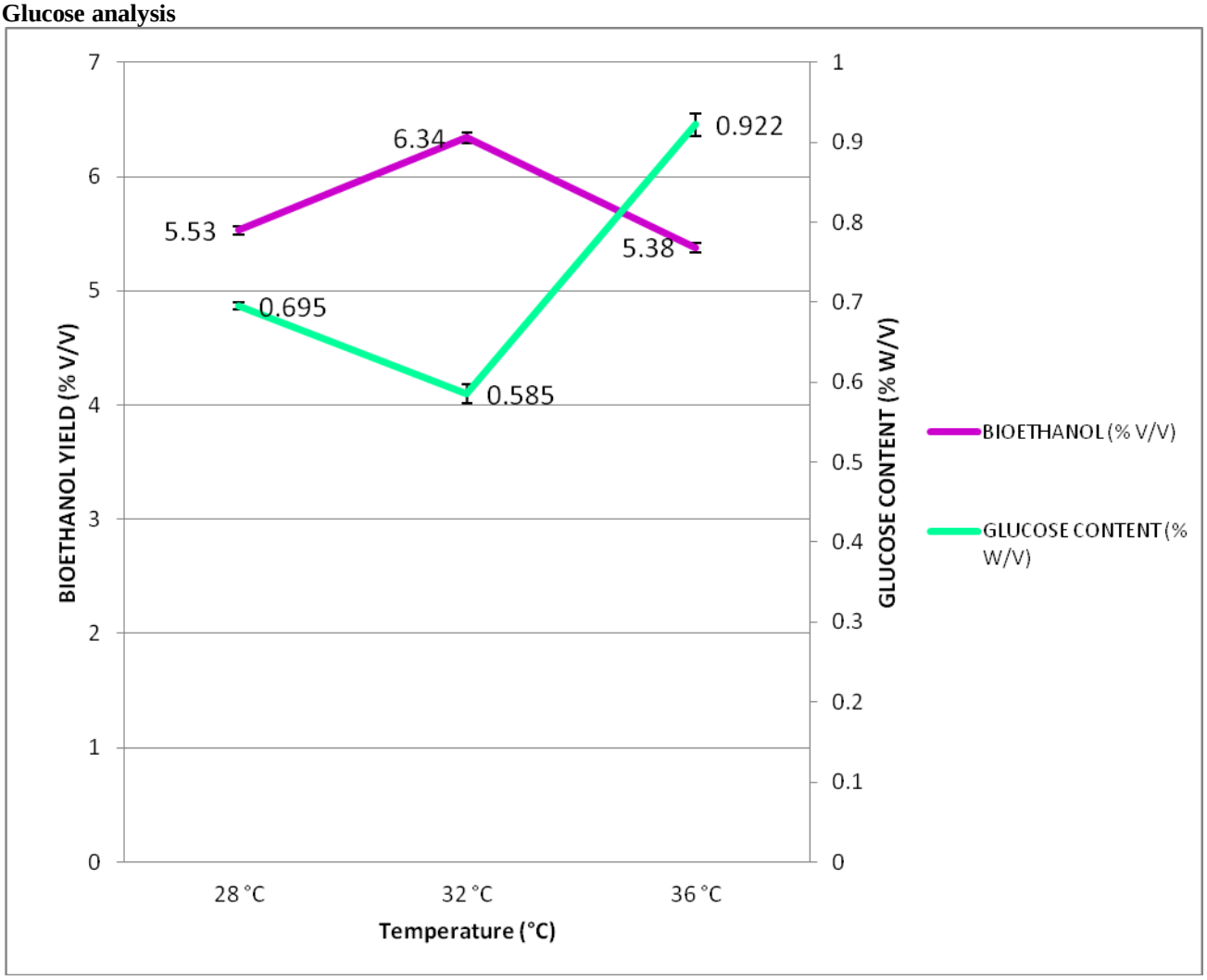

Figure 9: Glucose analysis of bioethanol

From figure 19, glucose content was lowest at 32°C, 0.585% (w/v) and this was correlated with high bioethanol, 6.34% (v/v). At 28°C, glucose was 0.695% (w/v) while bioethanol was 5.53% (v/v). At 36°C, the highest glucose was reported, 0.922% (w/v) and the bioethanol was lowest of all three, 5.38% (v/v).

Discussion

Effect of temperature on bioethanol production

Temperature had profound effect on *S. cerevisiae* growth. Sree *et al.*, (2000) reported the optimum temperature was between 25° C to 30° C. Liu, Lib & Shen (2008) published that fermentation at 32° C for 48 hours yielded the highest bioethanol from Sweet Sorghum. At low temperature, 28°C, cells were inactive and longer lag phase was obtained. Thus less ethanol produced by fermentation of glucose to give CO_2 as by-products. At 32°C, cells were at their most active form. Sugar consumption and alcohol production were greater. They were active and have short lag phase and normal log, stationary and death phase. Secondary metabolites to alcoholic fermentation increased as the temperature increased thus bioethanol yield was greater at 32°C. However, Wilkins *et al.*, (2007) studied fermentation of citrus peel by *S. cerevisiae* at 37° C while Slaa *et al.*, (2009) did at 35° C and both reported that was the best for bioethanol production.

Effect of pH on bioethanol production

Sachharomyces cerevisiae excel in pH 4 to 6 but they can survive in pH 2.5-8.5 (Narendranath, 2005). From Figure 10, it was clearly shown that pH 5 had the most yield of bioethanol, compared to pH 6 and pH 4. This result was

supported by Jovana *et al.,* (2009). Fermentation efficiency remained more or less same over the pH range of 5.0–6.0, and decreased marginally above 6.0 (Mohanty *et al.*, 2009).

Effect of fruit part on bioethanol production

Fermentation was done for 48 hours and the best result was by fermenting apple juice. Fuji apple has 9-11% sugar and 5 gram fiber by weight. Sugar consists mainly of glucose, sucrose and fructose and most of it remained in the juice, while fiber (cellulose, starch and hemicelluloses) remained in the pomace after extraction. *S. cerevisiae* is spectacular in fermenting readily sugar to CO_2 and ethanol. Juice contained high moisture and water compared with pomace and mixture. This resulted in better yeast activity to breakdown glucose thus yeast can easily access the substrate without need of pretreatment. Fermentation using apple juice yielded 7.05% bioethanol while mixture yielded 6.41%. Mixture consists of peel, pulp, calyx and seed or to be exacted, the whole fruit. Mixture has comparably high amount of sugar, starch and cellulose.

Apple pomace is biomass left after extraction. They consist of 25 to 30% of the weight of fresh fruit. From Figure 4, pomace had lowest bioethanol yield, only 4.19 % (v/v). This was due to low sugar content and high amount of polysaccharides (starch, hemicellulose and cellulose). Yeast could not breakdown starch and cellulose into simple sugar thus bioethanol yield was lower. Other reason for low bioethanol yield of apple pomace was because of low moisture level.

Effect of fruit condition on bioethanol production

Fermentation of rotten and fresh apple mixture was done for two days and from the Dichromate Colorimetric Method for Ethanol Assay (Williams & Reece, 1950 and Chatanta *et al.*, 2008), bioethanol yield was calculated. Yield was higher in rotten apple with 6.45% (v/v) while fresh produced only 5.65% (v/v) bioethanol. Bioethanol production from rotten fruit has been studied by Levey (2004) and Dudley (2002) and they found ethanol concentration increases as fruit progress from ripe to over-ripe to rotten.

Effect of enzyme hydrolysis on bioethanol produciton

Apple mixture contained pulp, peel, calyx and seed. Thus it had considerable high amount of sugar, starch and cellulose. *S. cerevisiae* can only ferment readily sugar and accumulation of ethanol cause reduction in yeast activity, cause low bioethanol yield, 5.87% v/v. To have better fermentation of apple mixture, a pretreatment is required before yeast inoculation to reduce the size, open up the plant structure and to ensure all polysaccharide had been broken down to monomers for yeast to utilize. There are two methods to optimized fermentation of lignocelluloses materials. The first was Separate Hydrolysis and Fermentation (SHF) and the second and most popular was Simultaneous Saccharification and Fermentation (SSF). Chatanta *et al.* (2008) explained SSF system of apple pomace by using mixture of microorganism of *S. cerevisiae, A. foetidus* and *F. oxysporum* for production of ethanol, pectinase and cellulase respectively. The combinations of all three strains result in 16.9% (v/w of apple pomace) ethanol with a residual sugar of 0.15% (w/w of apple pomace). SSF were done by mixing yeast with α-amylase for starch breakdown and with celulase for cellulose breakdown in separate Schott Bottles.

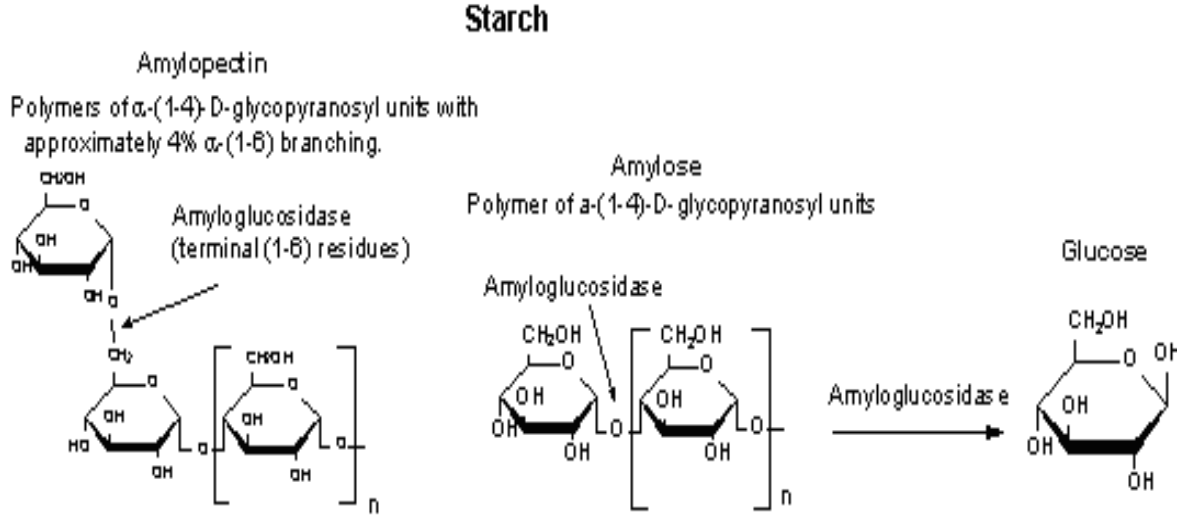

Figure 10: Starch hydrolysis by α-amylase (http://users.rcn.com)

Cellulose molecule is an unbranched polymer of 1000 to 1 million D-glucose units, linked together with beta-1,4 glycosidic bonds. The process of breaking the glucosidic bonds that hold the glucose basic units together to form a large cellulose molecule is called *hydrolysis* because a water molecule must be supplied to render each broken bond inactive. In this experiment, cellulase from Aspergillus niger was used. Cellulase had also been produced from Trichoderma using apple pomace as substrate (Sun *et al.*, 2010). In the first step, beta-1,4 glucanase breaks the glucosidic linkage to *cellobiose*. Subsequently, this beta-1,4 glucosidic linkage is broken by beta-glucosidase to glucose. This result in high readily sugar for yeast fermentation to produce the highest bioethanol yield (6.78% v/v). The accumulation of ethanol in the fermenter did not inhibit cellulase action as much as high concentration of glucose. SSF was a good strategy for increasing the overall rate of cellulose to ethanol conversion (Lin and Tanaka, 2006) and bioethanol yield was higher than fermentation with yeast alone or mixture of yeast with α-amylase.

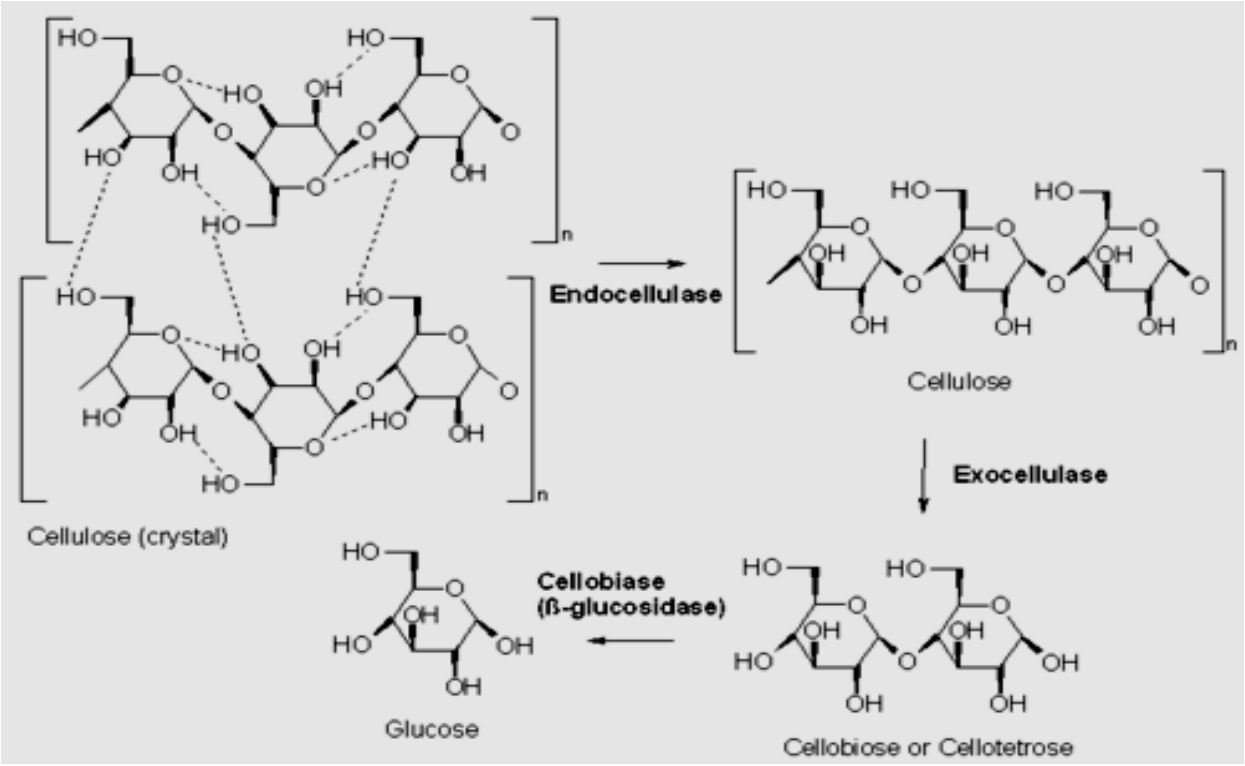

Figure 11: Process of cellulose hydrolysis to glucose

Analysis of bioethanol

Chemical analysis

From Table 1, shows the chemical elements value which come from the apple samples. If it is under the standards value, it will not be harmful in the industry level. According to the ASTM standard all value would be below 5ppm. Our results support the ASTM value.

Glucose analysis

Glucose assay was done using Dinitrosalicylic Colorimetric Method (Miller, 1959). The main purpose was to evaluate the concentration of reducing sugar, free carbonyl group (C=O), left inside bioethanol after fermentation. In fermentation process, one molecule of glucose is converted to two molecules of ethanol. Theoretically, for every kilogram of glucose fermented, 0.51kg ethanol and 0.49kg CO_2 shall be produced. This also proved for production of 1kg ethanol, 1.96 kg of sugars were needed. A studied conducted by Chatanta *et al*. (2008) on apple pomace showed when SSF was conducted with yeast for 72 hours, the initial sugar, 3.21% w/w dropped significantly to 0.25 % while the bioethanol yield rised to 8.44%.

The result showed at 32 °C, glucose content, 0.585 % (w/v) was much lower than ethanol, 6.34 %(v/v) . When bioethanol yield was highest, the glucose content was also lowest compared with 28°C and 36 °C. This indicated good fermentation process where most sugar had been utilized efficiently by *S. cerevisiae* to yield bioethanol. However, in this experiment, ethanol obtained was somehow reduced from the theoretical yield. This was because of incomplete fermentation of the sugar where small part of sugar was used by yeast to produce new cells and grow (Polycarpou, 2009).

Conclusion

It can be conducted that the best, parameters for bioethanol obtained were two days fermentation using 2g/l *S. cerevisiae* at 32 °C, pH 5 using rotten apple mixture. Bioethanol produced from fermentation of rotten apple biomass can be produced commercially as biosolvent in the laboratory, pharmaceutical, cosmetic, medical and biomedical industries level as substitute of ethanol and promote healthy waste management for a brighter future.

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