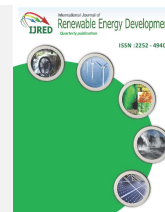




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Bioethanol Production from Iles-Iles (*Amorphophallus campanulatus*) Flour by Fermentation using *Zymomonas mobilis*

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ABSTRACT: Due to the depletion of fossil oil sources, Indonesia attempts to search new source of bioenergy including bioethanol. One of this sources is Iles-iles tubers (*Amorphophallus campanulatus*), which is abundantly available in Java Indonesia. The carbohydrate content in Iles-iles tuber flour was 77% and it can be converted to ethanol by three consecutive steps methods consist of liquefaction-saccharification using α and β -amylase, respectively and then followed by fermentation by using *Z. mobilis*. The objective of this research was to convert the Iles-iles flour to bioethanol by fermentation process with *Z. mobilis*. The ethanol production process was studied at various starch concentration 15-30% g/L, *Z. mobilis* concentration (10-40%) and pH fermentation of (4-6). The result showed that the yield of bioethanol (10.33%) was the highest at 25% starch concentration and 25% of *Z. mobilis* concentration. The optimum conditions was found at 4.5, 30°C, 10%, 120 h for pH, temperature, *Z. mobilis* concentration and fermentation time, respectively at which ACT tuber flour produced a maximum ethanol of 10.33 % v/v.

Keywords: alternative energy, bioethanol, ACT tuber, conventional, *Zymomonas mobilis*

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1. Introduction

The issues of fossil oil depletion have triggered Indonesia to search new renewable energy sources. Moreover, that issue is also stimulated by the increase of price of fossil fuels and the need to protect the planet from the climatic changes caused by the emission of greenhouse gases from burning them (Polycarpou, 2009; Aggarwal, 2007). Developments of new bioenergy such as bioethanol as alternative energy become a very paramount need.

Bioethanol (ethyl alcohol, grain alcohol, CH₃-CH₂-OH) is a liquid biofuel which can be produced from several different biomass feedstock and conversion technologies through fermentation using microorganism (Balat *et al.*, 2008). Bioethanol can be produced from various natural resources such as molasses (drops), starchy and lignocellulosic materials (Suresh, *et al.*, 1999). There are various feedstocks of

ethanol from starchy materials such as cassava, corn, sorghum grain, wheat, barley; from sugar plants such as sugarcane, sugar beet, sweet sorghum; from lignocellulosic materials such as wood and plant stems. 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, 2011). Disadvantages of bioethanol include its lower energy density than gasoline (<60%), its corrosiveness, low flame luminosity, lower vapor pressure, miscibility with water, and toxicity to ecosystems (Mac Lean & Lave, 2003).

In the last few decades, molasses has been considered as the main source of bioethanol production.

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However, the scarcity molasses as a raw material of bioethanol have need of new alternative raw material. The high demand of molasses for bioethanol as well as for animal feed and food additive industry has led to the increase of the price of molasses continuing to rise (Ferreira *et al.*, 2009). Although ethanol is mostly produced from molasses, however it is also produced from corn. Corn is used to produce 13 billion gallons of ethanol in the U.S. Starch containing in corn is a high yield feedstock for ethanol production, hydrolysis and fermentation steps are required to produce ethanol from starch material (Sa'nchez & Cardona, 2008). Another source for bioethanol is materials containing lignocelluloses despite of its lignin problem. The lignin in cellulosed materials can inhibit the action of the enzyme in breaking down polysaccharides during hydrolysis processes (Samsuri *et al.* 2007). Nevertheless, the productions of bioethanol from tubers and grains raw materials are still promising due to the abundant availability of starchy materials in Indonesia. One of the sources is *Amorphophallus campanulatus* or ACT tuber. *A. campanulatus* tuber (ACT) contains the lowest glucomannan (0-3%), while the highest of starch (77% starch), also another components at low concentration such as fiber (8.5%), crude protein (14%), reducing sugar (3-5%), ash and vitamins (3.4-5.3%). The main components of ACT tuber are 71.25% of starch, 8.54% of cellulose and 43.30% of hemicelluloses.

Zymomonas mobilis is a facultative anaerobic bacterium that able to utilize sucrose, glucose and fructose as an energy source, it has a high growth rate and resistance to ethanol concentrations of approximately 16% (Choi *et al.*, 2008). These bacteria can convert sugars to ethanol and CO₂ via the glycolytic "Entner-Doudoroff", where oxygen does not involve in the process of fermentation to avoid inhibition of respiration (Choi *et al.*, 2008). *Z. mobilis* is a type of bacteria which has an ethanol-tolerant characteristic, therefore it has the higher level of glucose uptake and able to produce ethanol in anaerobic conditions. In addition, these bacteria also have the ability to increase the ethanol purity (Rogers *et al.*, 2007). Furthermore, Choi *et al.* (2008) showed a great potential of this microorganism for conversion of industrial starch feedstock such as rice, barley, and cassava to produce bioethanol. In addition, Zang & Feng (2010) revealed that sweet potatoes at substrate concentration of 300-400 g/L fermented by using *Z. mobilis* could produced ethanol yield of 99.1-92.4%. Some studies showed that *Zymomonas mobilis* had a low energy efficiency resulting in a higher ethanol yield (up to 97% of theoretical maximum). *Z. mobilis* resulted the high ethanol content in the broth as in the case of very high gravity fermentations. *Z. mobilis* also had been used for ethanol production from dry-milled corn starch, in a system of continuous fluidized bed reactor with immobilized *Z. mobilis* which showed a high ethanol

concentration of 70 g/L and a productivity of 23 g/(L.h). Based on these great potential of *Z. mobilis*, the conversion of ACT flour to bioethanol was expected to be increased. The aim of this study was to utilize ACT flour as raw material for bioethanol production and facilitating *Z. mobilis* as microorganism in the fermentation. The separated saccharification and fermentation (SSF) method with α -amylase in the liquefaction and glucoamylase as enzyme at saccharification was employed to convert the ACT flour to bioethanol.

2. Materials and Methods

2.1 Preparation of *Z. mobilis* in solid and liquid media

The bacteria used in this study was *Z. mobilis* ATCC 29 191, these bacteria were inoculated in solid PGY medium containing 7.5 g of peptone, 20 g of glucose, yeast and 4.5 g of extract, 12 g of agar, then sterilized in autoclave for 15 minutes.

Z. mobilis was inoculated in a liquid medium containing 20 g of glucose, 1 g yeast, 0.5 g peptone, 0.1 g (NH₄)₂SO₄, 0.2 g KH₂PO₄, 0.05 g and 0.05 g FeSO₄ KH₂SO₄·7H₂O (Merck) in 100 mL of distilled water. The medium was sterilized at 121°C for 15 min.

2.2 Preparation of Raw Material

Fresh ACT tuber was cleaned, stripped and washed to remove the dirty particles. It was cut to small size (\pm 5 cm), dried under the sun shine until the maximum moisture content was \pm 10%. The dry ACT tuber was ground to 40 meshes to obtain ACT flour.

2.3 Hydrolysis

Hydrolysis enzyme consists of 2 stages, the first stage was liquefaction of ACT flour becomes a complex sugar and the second stage was saccharification. At the first stage, ACT flour was dissolved in water at different flour concentrations of 16, 20, 25, 30, 35 % (w/v). The liquefaction was carried out at pH 6 and 1.6% of α -amylase concentration per weight of ACT flour (v/w), temperature at 95-100°C for 1 hour. Then, saccharification of liquefied starch was performed at pH 5 by using 1.6% of β -amylase concentration per weight of ACT flour (v/w) and it was heated at 60°C for 4 hours.

2.4 Fermentation by using *Z. mobilis*

Prior to fermentation, the substrates that was placed in erlenmeyers were sterilized in an autoclave 121°C for 15 min. The fermentation was carried out using *Z. mobilis* at temperature of 30°C for 120 hours, the samples were withdrawn at 0, 24, 36, 48, 72, 96, and 120 min for remaining glucose and ethanol analysis.

2.5 Preparation ACT flour at various slurry concentrations

ACT flour was dissolved in distilled water at different substrate concentration of 16, 20, 25, 30, 35 %

(w/v). The substrate was liquefied, saccharified and followed by fermented by using 10% of *Z. mobilis* with adding nutrients at 0.2 gram of DAP and 0.4 grams of urea under pH of 4.5.

2.6 Preparation of fermentation slurry at various concentration of *Z. mobilis*

Fermentation of substrate slurry with different concentrations of *Z. mobilis* 10, 20, 30 and 40% (v/v) were prepared in a substrate slurry with ratio of substrate and water of 1:4. The slurry was subsequently added with 0.2 g of DAP and 0.4 g of urea to each sample and it was fermented at pH 4.5.

2.7 Preparation of fermentation slurry at various pH conditions

Fermentation was carried out at a substrate concentration ratio of 1:4, then it was added with 10% *Z. mobilis*, 0.2 g of DAP and 0.4 g of urea then fermented at pH 4, 4.5, 5 and 6.

2.8 Analysis

Glucose from hydrolysis was analyzed by the method of double sugar. 10 g of samples at the stage of saccharification with α -amylase and liquefaction with β -amylase was taken, and then 10 ml of HCl was added and boiled. After the solutions being cold, a 50 mL of distill water was added together with 4 drop of indicator phenolphthaline and neutralized with NaOH and diluted. The next step was boiling 5 mL of Fehling A, 5 mL of Fehling B and 3 drops of methylene blue, then titrated with a solution of the sample until the blue color disappeared. Reducing sugar resulted from fermentation was analyzed by the method of Somogy (1945). The analysis starts with mixing a 30 g of sample with 4 mL of 10 N HCl and boiled, then dissolved in 100 mL flask. Samples were taken 20 mL and neutralize using NaOH and diluted in 100 mL flask. One mL of sample was taken and 10 mL of copper reagent was added, and then put in a water bath at a temperature of 10°C, added 5 ml of sulfuric acid, then titrated with 0.005 N sodium thiosulfate until the color changes to light yellow, add 2 drops of starch indicator and continue titration until the color changes become clear. The reduced sugar was calculated by using Eq. 1.

$$\text{Glucose content (X)} = [A/B](Y) + [1/B] \quad (1)$$

Which, X is a glucose content (%), A and B is a reagent factor and Y is volume of titrate (mL)

3. Results and Discussion

3.1 Effect of various substrate concentration on the amount of reducing sugar

Carbohydrate content in ACT tuber was converted into glucose through hydrolysis of the two-step process which consists of liquefaction and saccharification.

Degradation occurs very rapidly and followed by a very rapid decrease in viscosity, whereas the second phase was much slower to form glucose and maltose. Enzyme α -amylase was used in starch hydrolysis liquefaction, while enzyme β -amylase was used in saccharification. α -amylase plays a role in breaking the bonds of α -1,4 glucoside from amylose and amylopectin, randomly. While β -amylase plays a role in breaking the bonds of glucoside (α -1,4 and β -1,6 glucoside) which available in the starch, dextrin and polysaccharide into glucose..

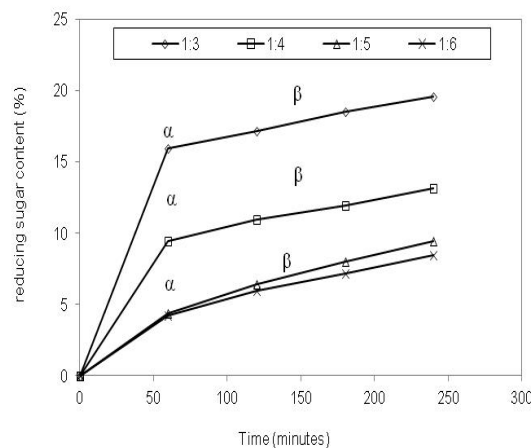


Figure 1 Effect of ACT tuber : water ratio on concentration of reducing sugar. Liquefaction conditions at α -amylase = 0.48 mL/g dry ACT tuber, T=100°C, t=1 h, pH 6 and Saccharification conditions at β -amylase = 0.48 mL/g dry ACT tuber, T=60°C, t=3 h, pH 5 were used

Figure 1 shows that the highest reducing sugar content was achieved at the ACT tuber: water ratio of 1:3, which resulted reducing sugar of 15.94% at one hour of liquefaction and 19.54% sugar at β -amylase at 4 hour saccharification. With increasing saccharification time reducing sugar was increased. This result was due to the more active side of the bound enzyme on the substrate that caused more glucose was formed. Substrates with a ratio of 1:3 having a minimum water content, therefore glucose was slightly dissolved in water, the more water content in the reactants would decreased of reducing sugar content. This fact was corresponded to the results shown in the concentrations of ACT tuber : water with ratio of 1:4, 1:5 and 1:6, which indicated the decreased in the result of saccharification of 13.33%, 9.45% and 8.45%, respectively. The result was in accordance with the study reported by Rani *et al.* (2010) which carried out bioethanol production from slurry of potato flour with solid : liquid ratio of 1:4. The potato tubers (*Solanum tuberosum*) flour was prepared by cooking and drying at 70°C prior to hydrolysis. The slurry of potato flour hydrolysis was then liquefied by using α -amylase at 80°C for 30 min and followed by saccharification using with *glucoamylase* at 60°C for 2 h which generated 15.2% total reducing sugars. Then, fermentation of

hydrolysate with *Saccharomyces cerevisiae* HAU-1 at 30°C for 48 h resulted in ethanol production of 56.8 g/l ethanol. Previous study conducted by Zang & Feng (2010) reported that at cassava substrate with concentration of 300 g/L produced the highest levels of 17.2% of reducing sugar content. This result corresponds to results obtained in this study, where the ratio of ACT tuber : water at 1:3 resulted the highest reducing sugar content of 19.54%.

3.2 Effect of ACT tuber suspension at various concentrations on Ethanol produced

Figure 2 shows the concentration of ethanol produced from the fermentation process with a variety of flour : water ratio in slurry. Ethanol concentration obtained were 7.4, 8.6, 6.0 and 5.22% (v/v) for the ratio of flour : water at 1:3, 1:4, 1:5 and 1:6, respectively which resulted yields of 38.36, 64.32, 63.92 and 43.24%, respectively. The highest ethanol concentration was shown in the ratio of flour : water at 1:4. The excessive solid flour or water content in the slurry resulted the slower reaction which could be shown in slurry at flour : water ratio below and above at 1:4.

In this study, the highest ethanol concentration (13.13% v/v) and yield (64.42%) were shown at flour : water ratio of 1:4 with fermentation time of 120 hours. These results were in accordance to previous research conducted by Zhang and Feng (2010) which converted glucose into ethanol by bacteria *Z. mobilis* which obtained ethanol concentration of 12.42% and yield of 61% in the presence of 200 g/L glucose. The use of *Z. mobilis* CICC10232 for bioethanol production has been evaluated for the fermentation of glucose at different concentration which showed that *Z. mobilis* CICC10232 could grow well in the glucose fermentation substrate with a concentration of 200-300 g/L glucose. The results indicated that at glucose concentration of 200, 250 and 300 g/L had the different length of lag period in 12, 24 and 36 h, respectively. In the presence of 200 g/l glucose showed the maximum ethanol yield (72.5 g/l) however the ethanol productivity was reduced in the presence of 250 g/L of glucose.

According to Zhang & Feng (2010), a high ethanol yield was produced from fermentation process at a high concentration of media, but to achieve a high yield it took a long time, therefore it had a low ethanol productivity. This result was in accordance with the experimental results which produced the highest sugar at ratio of flour : water at 1:3 which reached reducing sugar of 19.94%. The sugar concentration at ratio flour : water of 1: 4 was higher than that of 1:3 which produced 13.13% reducing sugar (as shown in Figure 1), however the highest ethanol yield was obtained at ratio flour : water of 1:4 which produced a yield of 64.42%.

The highest ethanol concentration at flour : water ratio 1:3 was due to the high density of

fermentation substrat therefore *Z. mobilis* bacterium could be more active. At the the ratio flour : water of 1:5 and 1:6, there were excessive water content in the substrate which resulted in a lot of glucose dissolved in water therefore *Z. mobilis* used a little glucose which converted into ethanol.

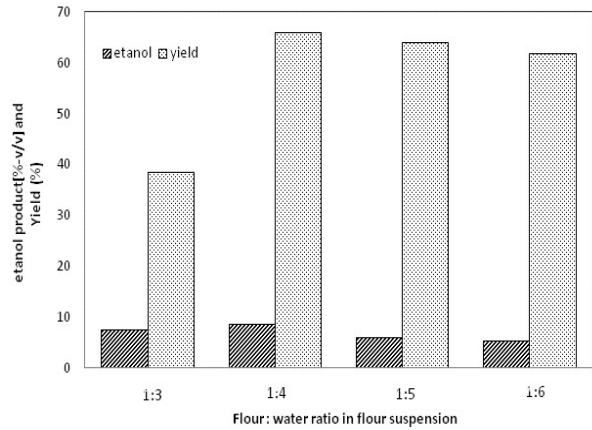


Figure 2 The effect of flour : water ratio in flour slurry on the ethanol concentration (% v/v) and ethanol yield. Hydrolysis conditions were constant at α -amylase = 0.16 mL/100 g dry starch, T=100°C, t=1 h, pH 6, β -amylase = 0.16 mL/100 g dry starch, T=60°C, t=3 h, pH 5. Fermentation condition was constant at 10% *Z. mobilis* pH 4.5, t=120 hours, T=28-30°C.

The effect of starch concentration had been evaluated which reported that the initial dissolved solids concentration exceeds 200 g/L implying a higher substrate load had been leading to a higher ethanol concentrations obtained. At a higher starch substrate, the lower amounts of process water was required and energy costs were decreased, however the drawbacks of high starch concentration had effected to a longer fermentation times and sometimes incomplete fermentations which probably caused by product inhibition, high osmotic pressures and inadequate nutrition.

3.3. Effect of *Z. mobilis* Concentration on Ethanol Produced

Figure 3 shows the ethanol concentration (% v/v) at various *Z. mobilis* concentration of 10-40% in fermentation substrate which produced ethanol of 8.45-10.16% for 120 hours fermentation. The highest ethanol content was reached in the concentration of *Z. mobilis* at 30% which resulted ethanol of 10.16% (% v/v).

The increment of ethanol concentration was in accordance with decreased in reducing sugar concentration, this was indicated that the consumption of sugar by *Z. mobilis* during substrate fermentation process. The rapid decreased in sugar fermentation occurred at 72 to 96 hours, from 3.37 to 0.19%, this condition was accordance with the increasing of ethanol content from 1.86 to 8.21 %. This indicated that *Z. mobilis* had a lag phase of 72 hours, the phase that

bacteria almost stopped growing and the its cell amount were likely to remain.

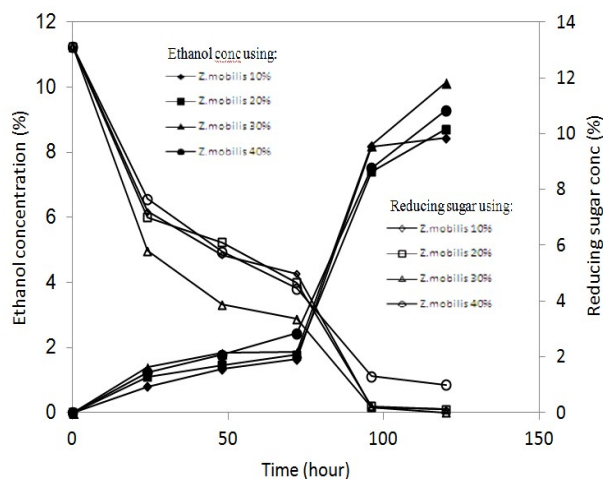


Figure 3 The ethanol concentration and reducing sugar resulted in fermentation on the various concentration of *Z. mobilis*. Fermentation condition was $T=28-30^{\circ}\text{C}$, $t=120\text{ h}$, $\text{pH } 4.5$, $\text{DAP}=0.2\text{ g}$, $\text{urea}=0.4\text{ g}$.

The lag phase of *Z. mobilis* shows very slowly compared with the *Saccharomyces cereviceae* that has 24-hour lag phase. The Log phase on *Z. mobilis* bacteria was occurred at 96 hours, because of its very rapid growth which characterized by a significant increased in ethanol concentration of 8.21%. The reducing sugar concentration from 96 to 120 hours fermentation was decreased slowly from 0.19 to 1.00%, because the most amount of sugar have been consumed by *Z. mobilis*. After the log phase, an increased the fermentation time led to the declined ethanol concentration due to a decreased in the number of bacteria that caused the amount of nutrients in the substrate decreased which resulted an increased the number of dead cells. This time was called the death phase. Polycarpou (2009) gained highest yield of ethanol at 30% of *Z. mobilis* but then it decreased after 40% *Z. mobilis*. This result was due to at the concentration of 40% *Z. mobilis*, the amount of sugar in the substrate was not completely converted to ethanol, but rather used as a carbon source for cell growth. The lower of ethanol yield from experimental compared to theoretical ethanol yield had been reported by Polycarpou (2009). The tuber called a *Asphodelus aestivus L* plant had been used for bioethanol production (Polycarpou, 2009). The process consisted of cooking the flour mash at a temperature of 95°C , combined by liquefaction and saccharification of the starch using enzymes, like alpha-amylase and glucoamylase. The starch content of the tubers resulted maximum bioethanol yield of 83.72 ml/kg tubers theoretically. However, from the experiments an ethanol yield of 49.52 ml/kg tubers was obtained. The reduction of ethanol due to the incomplete fermentation of the sugars in the mash during the three day

fermentation process. A small part of the sugar was used by the yeast to produce new cells and grow.

The higher concentration of *Z. mobilis* caused the higher concentration of ethanol produced in fermentation processes. This was due to the highest amount of sugar in substrate media of *Z. mobilis* which contain of 200 g of glucose, therefore the highest sugar could be converted to ethanol by *Z. mobilis*.

3.4. Effect of pH in Fermentation toward Ethanol Concentration

Figure 4 shows the ethanol concentration produced from the fermentation process with a various pH conditions. Ethanol concentration obtained at pH 4, 4.5, 5 and 6 were 3.77, 8.45, 7.18 and 7.65%, respectively at 120 h fermentation. Ethanol concentration reached a maximum at pH 4.5 (8.45%) at 120 hour fermentation. The decrease in reducing sugar concentration was occurred at 72 to 96 hours of 4.98 to 0.12%. At fermentation time from 0 to 72 hours, the ethanol concentration were low which produced 0.81, 1.33 and 1.64% at 24, 48 and 72 hours, this fact showed that at this time *Z. mobilis* through a lag phase or phase of adaptation. This suggests that *Z. mobilis* began working t 72 hours.

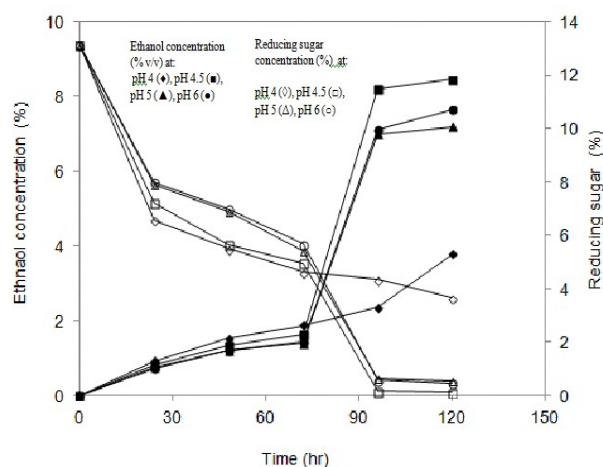


Figure 4 Ethanol and reducing sugar concentration from fermentation at different pH and constant conditions of $T=28-30^{\circ}\text{C}$, $t=120\text{ hours}$, $Z. mobilis=10\%$, $\text{DAP}=0.2\text{ g}$, 0.4 g urea .

Ethanol fermentation at the low pH of 4.5 using an acid-tolerant mutant of *Zymomonas mobilis* under non-sterilized condition had been evaluated (Struch *et al.*, 1991). At this low pH, potential contamination of ethanol production was controlled at with and without autoclaving or sterilization. The results revealed that compared with the sterilization process, the same ethanol yield of 73.1 g/l was obtained indicated that there were no contamination occurred in the non-sterilized process. At with the autoclaved process, ethanol yield increased from 70.3 to 73.2 g/l and residual sugar concentration decreased from 5.3 to 1.3

g/l for 40 h fermentation. The theoretical yield of ethanol from glucose was 0.488 g/g in this ethanol process.

The fermentation process worked well at low pH to minimize the contamination during the growth of bacteria, the more alkaline conditions led to the rate of fermentation process to convert bacterial to ethanol became slowly. At pH 4 ethanol produced was very low (3.77%) at 120 hours, this result was caused by too acidic medium condition which led to *Z. mobilis*, changed its chemical composition and its size therefore the increase in intracellular blocked. At pH 5 and 6 concentration of ethanol were 7.18 and 7.66%, respectively which caused by the high alkalinity condition of the fermentation media, therefore the rate of bacteria in converting sugar to ethanol was slow (Sun & Cheng, 2002; Suresh *et al.*, 1999). At pH 4.5, the percentage of ethanol concentration was highest therefore for further experiment, pH 4.5 was chosen as the best condition in fermentation process.

4. Conclusion

It could be concluded that ACT tuber is an attractive raw material for the production of bioethanol. Based on experimental results, the highest reducing sugar result in hydrolysis was obtained at a ratio of 1:3 which reached a sugar concentration of 19.54% at liquefaction by using α -amylase at 1 hour and saccharification by using β -amylase of 4 hours. Furthermore, the results of saccharified results fermentation of ACT tuber for 120 hours at various flours: water ratio, at the ratio of 1:4 yield ethanol concentrations of 64.32% (% v/v). In conclusion, ACT tuber could be a alternative new source of carbohydrate for bioethanol production by using bacteria of *Z. mobilis*.

Acknowledgments

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