Optimization of Glucose as Source of Bioethanol from Reed (*Imperatacylindrica*) Using Cellulase Enzyme at Various pH and Substrate Particle Size

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Abstract. Due to increasing demand of petroleum and limited source of fossil fuel, it is needed to explore and find source of a renewable energy. The biomass from agricultural residues, forest and farm waste has been known as a great source of energy and can be converted into biogas, bioethanol, biodiesel, etc. Reed which is abundant in Indonesia is one of potential sources of lignocellulose biomass that can be utilized as (*Imperata cylindrica*) a source of renewable energy such as bioethanol. The aim of this study was to optimize yield of glucose from reed using cellulose enzyme. Factors used in this research werepH and substrate particle size. Reed particle sizes were 80 and 100 mesh and pH of the solution were 4.2, 4.8 and 5.5. The results showedthat dried reed with100 meshes in particle size which hydrolysed in enzyme solution with pH 5.5 at 37^{o}C for 72 hours, producedthe highest yield of glucose in the amount of 80.2 mg/ml (8.02 %). When the incubation time was extended, glucose yield increased to 121.8 mg/ml (12.1%) at 96 hours and then decreased as the incubation time was extended further.
Keywords: Reed, Glucose, Bioethanol, Particle Size, pH

Introduction

One of the most serious problems faced by many countries in the world is rapid reduction in the availability of fossil energies. On the otherhand demand forfossil fuel, as the main source of energy, keep increasing, so that its price is soaring and the depletion rate is also increasing significantly. Production of ethanol by fermentation of sugar has already been commercially established but innovative studies could bring improvements to reactors and separation systems. Some sugars can be converted directly to bioethanol, whereas starch and cellulose must first be hydrolysed to sugar before conversion to bioethanol. Most of the polymeric raw materials are available at priceslower than refined sugars. However, transportation costs of the raw materials make it necessary to use locally available raw material (Taherzadeh, 1999).

To produce ethanol from lignocellosic materials, it is essential to hydrolyse it before fermentation at the demonstration and industrial level. Enzymatic hydrolysis is still at an early stage, requiring substantial fundamental research (e.g., for increased yields) (Kucuk and Demirbas, 1997). Therefore, there is a need to explore and develop an alternative source of energy, especially a renewable energy such as biomass, a bio-based energy. The main sources of biomass are forest and agricultural residues, grass, and waste from farm and industry such as hay, dry grass, and bagasse. Biomass can be converted into a number of different types of energy such as biogas, bioethanol, biodiesel, bricket, etc.

Reed (*Imperatacylindrica*) is a kind of grass with sharp long leaves that grow rapidly in an agricultural land and considered as a weed by farmers. This grass contain a significant amount of cellulose and can become one of the source of lignocellulose biomass that can be converted into bioethanol. Study advances in thermal processing of cellulose may offer a new alternative for biomass pre-treatment and saccharification (Shafizadeh and Stevenson, 1982; Piskorz *et al.*, 1989; Radlein *et al.*, 1991).The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulose activity, and reaction conditions (temperature, pH, as well as other parameters). To improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the hydrolysis process and enhancing cellulose activity (Cantwell *et al.*, 1988; Durand *et al.*, 1988; Orpin,

1988).Indarti *et al.* (2012) reported, using 30 mesh reedpowder, found that glucose hydrolysed from the grass using cellulose enzyme was only 53.5 mg/ml, which was considered low. Several factors that might cause the low yield were relatively large substrate particle size and decreasing pH during hydrolysis incubation. At 30 mesh particle size, enzyme might not work optimally, due to less surface area available that allowing optimal contact between enzyme active site and the substrate surface. Therefore, in this research, the grass particle sizes were reduced further to 80 mesh and 100 mesh.

To keep its activity high, enzyme environment needs to be maintained properly. Indarti *et al.* (2012)reported that the pH of the solution decreased gradually as the hydrolysis progressed. Some researcher reported that cellulose enzyme work better in the solution with pH about 4 (Diyono *et al.* 2008), 4.8 (Bill andBaker, 1996) and 5-7 (Lehningher, 1995). However, optimum pH value for an enzyme might differ from one process to another. Therefore, in this research, optimum pH value of the fermentation solution was explored by controlling the pH values in the solution at certain level through the fermentation process. The solution pH was set to 4.2, 4.8 and 5.5.

Materials and Methods

Materials and equipments

The main material used in this research wasreed (*Imperatacylindrica*) harvested from the field in Cot NamBak Village, Sub District KutaMalaka, in District Aceh Besar, Indonesia. The enzyme used was isolated form fungy*Trichodermareesei*produced by Sigma Aldrich-USA (C8546-5KU). Chemicals used in this research were aquadest, tween 80, 1 M citric acid buffer, and 1 N potassium hydroxide (NaOH), while chemicals for analytical purposes were anthrone solution, H_2SO_4 97 %, alcohol 96%, pure glucose and aquadest.Equipment used in the research were grinder, hammer mill, steam reactor, laminar flow cabinet, incubator, autoclave, analytical balance, thermometer, glassware, micro pipet, and screen with 80 and 100 mesh in sizes.

Experimental design

The factors used in this experimental were pH and particle size of substrate (reed). pH of the substrate solution used were 4.2, 4.8 and 5.5 and particle sizes of biomass were 80 and 100 mesh.Glucose was analysed using Anthrone method (Apriyantono, 1989). The correlation betweenreed particle size and pH on glucose obtained was performed in a graph. Optimization was identified by the highest glucose obtained. Glucose was analysed during hydrolysis in 2, 4, 6, 8, 12, 24, 48 and 72 hours. If the glucose production still increased after 72 hours, the best hydrolysis condition (basedon pH and reed particle size) would re-conducted in the second phase to find the highest glucose yield.Glucose concentration were then analysed at 94 till 140 hours. Optimum condition was identifiedby the highest glucose production

Cellulose preparation

Reed (*Imperatacylindrica*) was harvested and sun dried for 4-5 days. Size reduction was conducted by two steps. In the first step,dried reed was chopped using chopper to medium size (30-60 mesh) and in the second step,was milled using hammer mill up to 80 to 100 mesh size. 20 g of the reed powder was used as substrate for hydrolysis. Reed powder was soaked in 100 ml 2% of sulphuric acid solution, in 250 mL Erlenmeyerglass and leaved for 20 hours.In order to enhance the efficiency of glucose recovery from cellulose, lignin and hemicellulose must be separated from cellulose. The reed substrate was then hydrolysed in steam reactor with pressure of 2 bars for 10 minutes. Afterward the pressure was immediately decreased to 0.5 bars in and leaved until room temperature. The substrate then washed using 50^{o}C water and kept in 4^{o}C until hydrolysed (Indarti *et al.,* 2012).

Cellulose hydrolysis

The 20 grams of reed substrate was thenplaced into 250 ml Erlenmeyer glass and diluted in 100 ml aquadest and. pH of hydrolysis solution was controlled and set at 4.2, 4.8 and 5.5. Sterilization of the substratewas conducted in autoclave at 121^{o}C for 15 minute, and then leaved for natural cooling.When room temperature reached, 30 mg cellulose

enzyme and 0.05 g tween 80 were added into the substrate. Then the substrate solution was incubated in incubator at 37°C and pH was controlled by adding NaOH.

Analysis

Cellulose content was analysed before and after hydrolysis process. pH and glucose content were analysed during saccharification at 2, 4, 6, 8, 12, 24, 48 and 72 hours.

Results and Discussion

Cellulose content

Dried reed components comprise of lignocellulosic was treated by separating the cellulose from other major component hemicellulose and lignin. Thispre-treatment step usedNaOH 2% and a 2 bar pressure. Cellulose content from reed before pretreatment was about 31.6 - 32% and become higher after pre-treatment cellulose, which was 49 %. This was due to the pre-treatment process which caused the breaking of the licgnocellulosic bond to form cellulose, hemicellulose and lignin. Grous *et al.* (1986) reported that the pre-treatmentprocess causes hemicellulose degradationand lignin transformation due to high temperature, thusincreasing the potential of cellulose hydrolysis. Ninetypercent efficiency of enzymatic hydrolysis has beenachieved in 24 h for poplar chips pre-treated by steamexplosion, compared to only 15% hydrolysis of untreatedchips.

The effect of pre-treatment process of lignocellulosic materials has been recognized for a long time (McMillan, 1994). Dilute acid hydrolysis has been successfully developedfor pre-treatment of lignocellulosic materials. Thedilute sulphuric acid pre-treatment can achieve high reactionrates and significantly improve cellulose hydrolysis(Esteghlalian *et al.*, 1997). Dilute NaOH treatment oflignocellulosic materials caused swelling, leading to anincrease in internal surface area, a decrease in the degreeof polymerization, a decrease in crystallinity, separationof structural linkages between lignin and carbohydrates,and disruption of the lignin structure (Fan *et al.*, 1987).

Glucose yield

Glucose concentration had been analysed at certain times during hydrolysis process. Figure 1 shows glucose concentration at various pH and substrate particle size at different incubation time. Reed particle size of 80 meshes at pH 4.2; 4.8 and 5.5 resulted glucose of 30.9 mg/ml, 35.3 mg/ml and 52.5 mg/ml, respectively. Meanwhile, the one of 100 meshes at pH 4.2; 4.8 and 5.5 produced higher glucose content 45.4 mg/ml, 54.4 mg/ml and 80.2 mg/ml, respectively. It showed that the higher the pH condition, the higher glucose concentration obtained. Similarly the reed particle size affected the glucose production. The trend of higher glucose production continued up to 72 hours of Hydrolysis process.

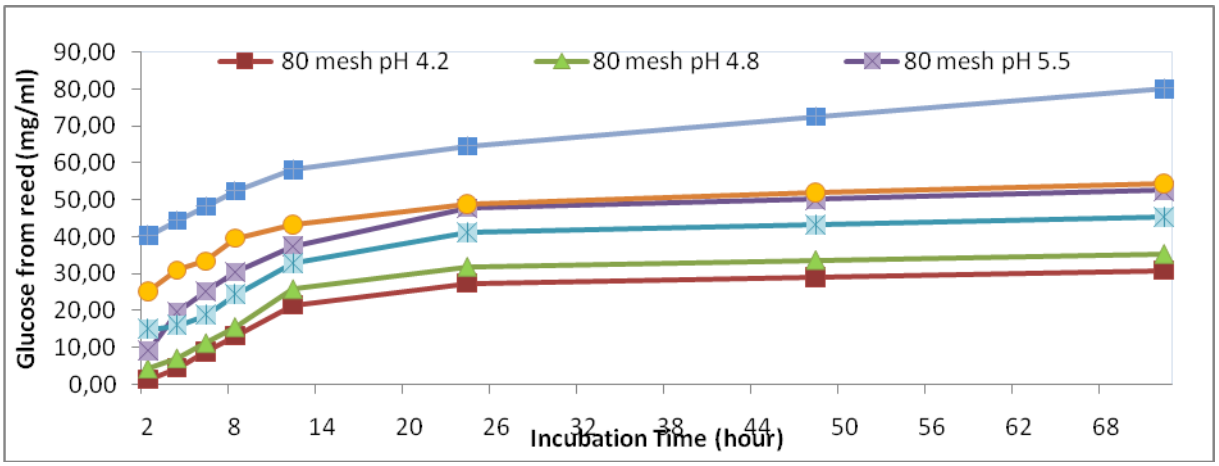

Figure 1Glucose production from reed at various pH and substrate particle size.

Enzymatic hydrolysis of cellulose is carried out by cellulose enzymes which are highly specific (Beguin and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. However during hydrolysis process cellulose activity may inhibited by cellobiose and to a lesser extent by glucose. Several methods have been

developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of b-glucosidases during hydrolysis, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (SSF).The SSF process has been extensively studied to reduce the inhibition of end products of hydrolysis (Takagi *et al.*, 1977; Blotkamp *et al.*, 1978; Szczodrak and Targonski, 1989; Saxena *et al.*, 1992; Philippidis *et al.*, 1993; Zheng *et al.*, 1998). In the process, reducing sugars produced in cellulose hydrolysis or saccharification is simultaneously fermented to ethanol, which greatly reduces the product inhibition to the hydrolysis.

In this research pH 4.8 was conducted at 37°C. Figure 1 showed that increasing the pH up to the pH 5.5 at the same temperature resulted in higher glucose content. Meanwhile, Susilawati *et al.* (2002) reported that optimum condition activity of cellulose enzyme was held at pH 4.5 – 6.5. Lehningher (1995) also state that pH optimum for cellulose enzyme is 5 – 7. While Duff and Murray (1996) stated that the optimum condition was at pH 4.8 and temperature 45–50°C. In this research, the pH tend to decrease during hydrolysis time, therefore the pH was maintained by adding NaOH 0.1 N. Due to the optimum condition was not reached, the best hydrolysis condition of this stage was re-conducted for extended hydrolysis time up to the time when the glucose tend to decreased.

Optimization

The amounts of glucose produced from the hydrolysis process wereused to measure the cellulose yieldfrom each pH and substrate particle size condition. The wet residues wereweighed to find out the amount of cellulose left.

Tabel 1 shows that the higher the pH of solution, the higher the amount of glucose obtained. Similarly, the higher the mesh of particle size, the higher the glucose concentration produced. This was due to the surface area of 100 meshes higher than 80 mesh of particle size. Dian *et al.* (2007) reported that rate of saccharafication process using cellulose isolated from *Trichodermaviride* inbiomass of 200 mesh particle size increased 11% compared to the unreduced particle size. Reed hydrolysis processing using 100 mesh particle size and pH 5.5 resulted in glucose of 80.2 mg/ml (8.02 %), while the initial reed substrate was 20 g; this meant that the yield of this hydrolysis was 8.02 g/ 20 g equal to 40.1 %.

Table 1. Yield of glucose from reed.

Biomass Condition	Wet Residue (g)	Glucose (mg/ml)	Yield (%)
Reed: 100 mesh, pH 4.2	16	30.9	15.45
Reed: 100 mesh, pH 4.8	15.4	35.3	17.65
Reed: 100 mesh, pH 5.5	13.8	53.5	26.25
Reed: 100 mesh, pH 4.2	14.3	45.4	22.70
Reed: 100 mesh, pH 4.8	13.4	54.4	27.20
Reed: 100 mesh, pH 5.5	11.2	80.2	40.10

Second phase

Figure 1 shows that the trend of the graphs still increasing meant that the production of glucose still increase even after 72 hours. Therefore, the best condition of the first phase was re-conducted in the second phase of hydrolysis process. The process at pH 5,5 and 100 mesh reed particle size was conducted with the hydrolysis time prolonged until 140 hours. The graph of glucose production was shown in Figure 2.

After prolonged the hydrolysis time, glucose production increased up to121.8 mg/ml (12.1%) after 96 hours. However, the glucose concentration slightly decreased after optimum condition reached. This might due to the inhibition of enzyme activity by cellobiose and glucose. Use of a cellulose mixture from different microorganisms or a mixture of celluloses and other enzymes inthe hydrolysis of cellulosic materials has been extensively studied (Beldman *et al.*, 1988; Excoffier *et al.*, 1991; Xin *et al.*, 1993). The addition of b-glucosidases into the *T. reesei*cellulases system achieved better saccharification than the system without b-glucosidases(Excoffier *et al.*, 1991; Xin *et al.*, 1993). b-glucosidaseshydrolyse the cellobiose which is an inhibitor of cellulose activity. A mixture of hemicelluloses or pectinases with celluloses exhibited a significant increase in the extent of cellulose conversion (Ghose and Bisaria, 1979; Beldman *et al.*, 1984).

The result of glucose production after 96 hours of 12,1% in this study had fulfilled the requirement of glucose concentration as raw material for ethanolysiswhich require 10-18% glucose.

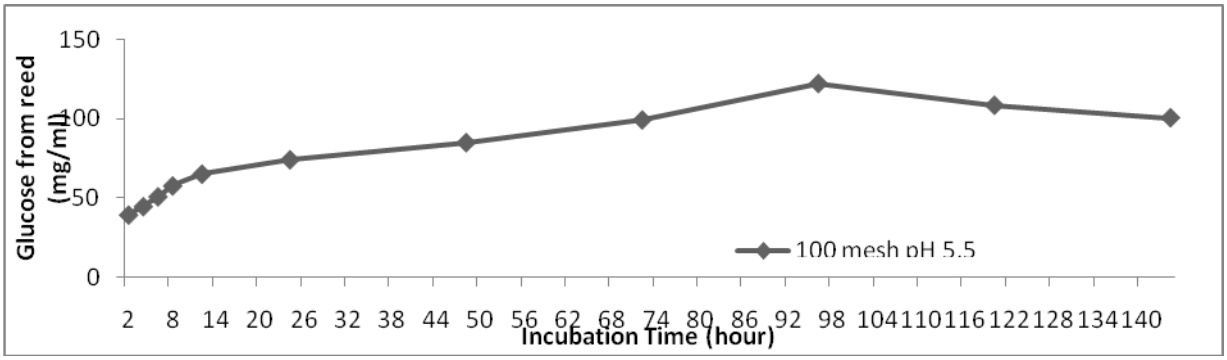

Figure 2. Glucose production at various hydrolysis time at 100 mesh particle size and pH 5,5.

Conclusion

In order to produce cellulose from reed, a pre-treatment process was used to break down the lignocelluloses to cellulose,reduce the sample size, and open up the structure of the cellulose component.Particle size of dried reed of 100 mesh produced higher glucose content compared to the 80 mesh. pHsubstrate solution of 5,5 resulted higher glucose concentration compared to other pH of solution (4.2 and 4.8). The best condition for hydrolysis processwas 100 mesh of reed particle size, pH solution of5,5 which resulted glucose of 121, 8 mg/ml at 96 hour of hydrolysis time

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