

Research Article

Ethanol production from cellulosic cassava waste

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Abstract

Cassava cellulosic waste obtained from starch processing was utilized in this work for bio-ethanol production. The 10% (w/v) of cassava waste was pretreated with either water or 1% (v/v) acetic acid and steam treated by autoclaving at 121 °C under pressure of 15 pound/inch² for 15 min. After neutralization of the acid-autoclaved pretreated waste with 2% (w/v) NaHCO₃, the mixture was hydrolyzed further by using 3% or 6% (w/w) cellulase for sugar production. It appeared that the optimum hydrolysis condition was 1% (v/v) acetic acid and 6% (w/w) enzyme (shaking at 50 °C, 150 rpm for 96 h), which gave the maximum sugar yield of 199.82 ± 0.27 mg/g dried cellulosic waste. A total of 515.39 ± 0.48 mg ethanol/g dried cellulosic waste was obtained from 9% (w/v) of *Saccharomyces cerevisiae* in 7-days fermentation at room temperature. This indicates that cassava wastes actually could be used as substrate for biochemical production, such as fuels, organic acids and etc. In addition, the pretreatment method using dilute acetic acid and steam is environmentally friendly and not complex.

Keywords: acetic acid, cassava waste, cellulase, fermentation

Introduction

Cassava is considered one of the most important economic crops in Thailand. Nearly all the harvested roots are processed into dry chips and pellets for export as animal feed, as well as into starch, both for domestic use and export (Ratanawaraha et al., 2000). Waste material from cassava-starch processing is about 1.1% of cassava tubers that enters the factory. This is approximately 2 million tons per year. If this waste is not properly managed, it may cause foul smell and unattractive sight (Sackey & Bani, 2007; Deeprasert et al., 2012). The technologies currently existing for treatment of this waste include land filling of solid waste, use as animal feed, utilization as a media for mushroom cultivation, or use to produce compost. Major components in this waste are cellulose and starch. If this material can be degraded and hydrolysed to sugar, it can be used as raw material to produce many value-added components; for example, lactic acid, ethanol, biogas and so on (Ubalua, 2007; Sackey & Bani, 2007).

A total of 32.4% alcohol (2.7 g ethanol/15 g cellulosic waste) was obtained from cassava cellulosic waste when the waste was first hydrolysed by 0.1% α-amylase, followed by hydrolysed using 0.5 M HCl. Finally, amyloglucosidase was used to saccharify the hydrolysed starch into reducing sugar (Elemike et al., 2015). Thongchul et al. (2015) reported that the maximum starch recovery (88%) and the highest glucose production (98 g/L) were obtained when hydrolyzing cassava pulp at 121°C for 15 min with 1.0 M HCl (1 g dry pulp to 9 mL HCl

solution). Recently, a soaking assisted thermal pretreatment was introduced for optimum fermentable sugar release from cassava peels waste. Maximum reducing sugar of 89.80 ± 2.87 g/L corresponding to a fermentable sugar yield of 0.93 ± 0.03 g/g cassava peels was achieved when 9.65% w/v of milled cassava peels were soaked in 3.68 v/v hydrochloric acid concentration at 69.62 °C for 2.57 h followed by autoclave thermal treatment (121 °C) for 5 min (Aruwajoye et al., 2017).

However, these works used strong acid in the pretreatment of the waste. Therefore, the aim of this report was to study the utilization of cassava cellulosic waste as raw material for ethanol production using weak acid (acetic acid) and cellulase in the pretreatment method. The factors that were investigated included the pretreatment condition, the concentration of cellulase, the hydrolysis time and the concentration of yeast used in the fermentation.

Materials and methods

Raw material

The cassava cellulosic waste used in this study was provided by Thai Wah Starch Public Company Limited, Nakhon Ratchasima Province. The waste was collected directly from the plant, milled into fine particles and dried at 100 °C for 24 h. Then, it was kept in moisture-proof bag at room temperature.

Proximate analysis

Some biochemical parameters such as crude fibre content, protein content and total carbon of the cellulosic cassava waste was determined. The protein content was determined by Kjeldahl method using the conversion factor of 6.25 (AOAC, 2012). The fibre content was also determined by using AOAC, 2012 method. The sample is allowed to boil with 1.25% dilute H_2SO_4 , washed with water, further boiled with 1.25% dilute sodium hydroxide and the remaining residue after digestion was taken as crude fibre. This fibre content was roughly estimated as cellulose content. Total carbon content was estimated based on the volatile solids (VS) content. % Carbon equal to (% VS) / 1.8 (Adams et al., 1951).

Microorganism

Saccharomyces cerevisiae used in this study was commercial instant dry bakers yeast, Fermipan Red.

Pretreatment

The wastes were pretreated by either water or 1% (v/v) acetic acid to increase the accessibility of cellulose, at solid to liquid ratio of 1 : 10 (w/v). The condition used was either incubation at room temperature for 24 h or steam-treatment by autoclaving at 121 °C and 15 pound/inch² pressure, with reaction time of 10, 15 and 20 min. Reaction mixture was allowed to cool to room temperature and 1 mL of hydrolysate was pipetted out to estimate the concentration of total reducing sugar (TRS) using dinitrosalicylic (DNS) acid method (Miller, 1959). Glucose was used as a standard in this assay. The best pretreatment that gave the highest reducing sugar was used further for enzyme hydrolysis.

Enzyme hydrolysis

The effects of enzyme concentration and hydrolytic conditions were evaluated by adjusted the pH of the acid-autoclaved pretreated waste to 5.5 using 2% (w/v) $NaHCO_3$ solution. The neutralized waste was then subjected to enzymatic hydrolysis using commercial

cellulase solution Cellic[®] CTec2 (Novozymes, Denmark). The wastes were hydrolyzed with 3% or 6% of Cellic[®] CTec2 (W enzyme/W waste) at 3 different condition for 48 h : stand at room temperature; shaking at 30°C, 150 rpm; and shaking at 50°C, 150 rpm. The samples were harvested at 6-h interval to assay for the concentration of TRS using dinitrosalicylic (DNS) acid method (Miller, 1959).

Ethanol fermentation

After enzymatic treatment for 48 h, the waste hydrolysate was separated into 2 groups, one was sterilized by carried out in an autoclave at 15 pound/inch² pressure, 121°C for 15 min, the other was not autoclaved. Then, they was inoculated with 3 different concentrations of yeast; 3%, 6% and 9% (w/v). Each experiment was carried out in duplicate. In addition, the extension of enzymatic treatment to 72 and 96 h, without sterilization of the waste hydrolysate before yeast inoculation, was also carried out. The samples were harvested each day to assay for the concentration of TRS using dinitrosalicylic (DNS) acid method (Miller, 1959), and ethanol using gas chromatograph with a 10% Carbowax 20 M support (6 ft. x 1/8 in), at oven temperature of 150°C and flame ionization detector (FID) at 200 °C. Nitrogen with a flowrate of 30 mL/min was used as carrier gas.

Results and discussion

Effect of pretreatment condition

Cassava cellulosic waste contained (wt. %): 2.33 protein, 13.30 cellulose and 58.04 total carbon. The composition of cellulose is in agreement with the previously reported values, 14.35% and 15.25% w/w dry basis (Yoonan, 2003; Deeprasert et al., 2012).

Most acid hydrolysis pretreatment for cellulosic material used concentrated strong acids such as H₂SO₄ and HCl. Although they are powerful agents for cellulose hydrolysis, concentrated strong acids are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pretreatment process very expensive. In addition, the concentrated strong acid must be recovered after hydrolysis to make the process economically feasible (Kumar et al., 2009).

Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. High temperature in the dilute-acid treatment is favorable for cellulose hydrolysis (Kumar et al., 2009). Furthermore, it was reported that the presence of weak acids can improve glucose utilization, ethanol production and tolerance to hydroxymethylfurfural and furfural in *S. cerevisiae* (Greetham et al., 2016). Therefore, acetic acid was used in this study to pretreat cassava waste compared to the use of water either at room temperature or in the autoclave.

According to Figure 1, the condition that gave the highest yield of reducing sugar was the pretreatment of cassava waste with 1% (v/v) acetic acid in autoclave at 121 °C for 15 min (22.08 ± 0.35 mg/g dry wt.) The value was not significantly different from that of the pretreatment with 1% (v/v) acetic acid at 121 °C for 20 min (21.08 ± 0.24 mg/g dry wt.). This condition was better than the condition that used only water in autoclave and only acetic acid at room temperature for 24 h (19.17 ± 0.00 and 4.76 ± 0.04 mg/g dry wt., respectively). This is due to the entrance of steam into the biomass expanding the walls of fibers leading to partial hydrolysis and increasing the accessibility of enzymes for cellulose. After this, the pressure is reduced to atmospheric condition. During this pretreatment, the hydrolysis of cellulose into glucose monomers is carried out by the acetic acid (Kumar & Sharma, 2017). Water can act as an acid at high temperatures. Addition of acid [typically 0.3-3% (w/w)] in steam explosion can

decrease time and temperature, effectively improve hydrolysis and decrease the production of inhibitory compounds. Steam provides an effective vehicle to rapidly heat cellulose to the target temperature without excessive dilution of the resulting sugars (Kumar et al., 2009). The pH of acid-autoclaved pretreated waste was adjusted to 5.5 by 2% (w/v) NaHCO₃ solution before using further.

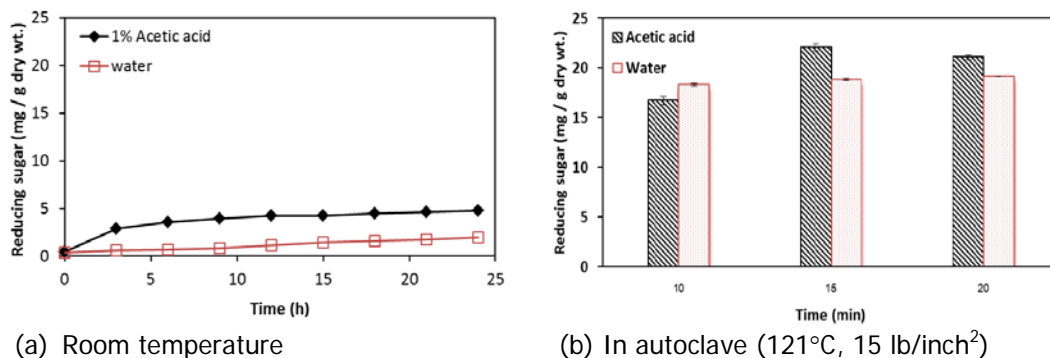


Figure 1. Effect of acetic acid concentration (0% and 1% (v/v)), incubation condition and time on the sugars yield of cassava waste.

Effect of enzyme loading

To increase the efficiency of the acid pretreatment, enzymatic hydrolysis was performed using the neutralized sample obtained after acid autoclaving. The different enzyme concentration (3% and 6% (w/w) Cellic[®] CTec2) and incubation temperature (room temperature, shaking at 30°C and 50°C) was evaluated. The highest yield of glucose (138.61 ± 0.37 mg/g dry wt.) was obtained from the enzymatic hydrolysis using 6% w enzyme/w of cassava waste, shaking at 50°C for 48 h, after acid autoclaving (Figure 2). On the other hand, 110-115 mg glucose/g dry wt. of cassava waste was obtained when 3% (w/w) cellulase was used at either 30°C or 50°C for 48 h. Increasing enzyme concentration gave higher sugar yield. This was similar to that reported by Bayitse et al. (2015). Enzymatic hydrolysis of cassava peels with 2.5% (v/w) cellulases (NS22186) at 4 h released 0.9 g/l of glucose which rose to 1.2 g/l in 48 h. When the enzyme loading was increased to 10% cellulase, glucose concentration was doubled from 1.02 g/l at 4 h to 2.17 g/l at 48 h of hydrolysis which increased marginally to 2.6 g/l at 120 h. The amount of fermentable sugar obtained increased as the enzyme load increased while cellulose load decreased (Azhar et al., 2017). Deeprasert et al. (2012) can produce 65.80 g/l of reducing sugar from cassava solid waste (100 g/L), using 3 steps of enzymatic hydrolysis, Cellic[®] CTec2 for 6 h, α-Amylase (Novozyme) for 2 h and Glucoamylase (Novozyme) for 12 h. However, the presence of high sugar concentration in the fermentation medium may lead to substrate inhibition and results in the inhibition of cell growth and ethanol production (Azhar et al., 2017).

In order to investigate the effect of the concentration of acetic acid in the pretreatment before enzyme hydrolysis, the acetic acid concentration was increased to 3% and 5% (v/v) before hydrolysed by 6% (w/w) Cellic[®] CTec2. The result showed that the sugar yield obtained from 3% and 5% acetic acid pretreatment was not significantly different from that of 1% acetic acid (Figure 3). Esteghlalian et al. (1997) found that high temperature in the dilute acid treatment is favourable for cellulose hydrolysis. Dilute sulfuric acid (<2% w/w) pretreatment can achieve high reaction rate and makes the cellulose more susceptible to enzymatic

digestion. Horst et al. (2011) reported that acetic acid showed to be potential used for lignocellulosic hydrolysis. The hydrolysis efficiency levels of acetic acid against several types of wood chips was maintained between 21.47% and 32.62%, and the ethanol production rates stayed between 6.32 L/100 kg and 9.63 L/100 kg of biomass. Zhao et al. (2014) found that pretreatment of corn stover with 0.25% acetic acid at 191 °C for 7.74 min was the most optimal condition under which the production of acids in acidogenic fermentation can reach the highest level. Schneider et al. (2016) mentioned that acetic acid and formic acid are rather mild acid yielding low total reducing sugar levels compared to the sulfuric acid, when it was used to convert lignocellulosic barley straw to valuable sugars.

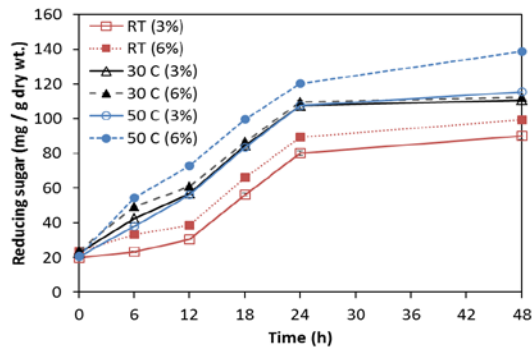


Figure 2. Effect of Cellic® CTec2 concentration (3% and 6% W enzyme/W cassava) and hydrolysis condition on the sugar yield of cassava waste pretreated with 1% (v/v) acetic acid in the autoclave for 15 min. The cellulolysis condition was stand at room temperature (RT), shaking at 30 °C (30 C) and 50 °C (50 C) for 48 h.

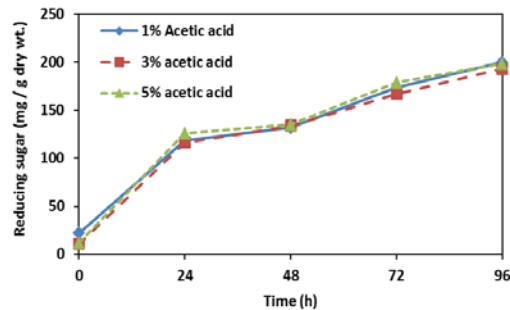


Figure 3. Effect of acetic acid concentration (1%, 3% and 5% (v/v)) in the autoclave for 15 min before enzyme hydrolysis on the sugar yield of cassava waste.

Furthermore, the longer the hydrolysis time was, the higher the sugar yield obtained. The sugar yield of 96-h hydrolysis of the cassava waste pretreated with 1%, 3% or 5% (v/v) acetic acid before enzyme hydrolysis were 199.82 ± 0.27 , 193.20 ± 1.20 and 198.61 ± 1.85 mg glucose/g dry wt; compared to 136.37 ± 2.76 , 133.97 ± 2.41 and 134.97 ± 2.33 mg glucose/g dry wt of 48-h hydrolysis, respectively (Figure 3). Therefore, the hydrolysis time may be more important than the acid concentration used. This was similar to the result of Bayitse et al. (2015), as mentioned above.

Ethanol fermentation

The acid-autoclaved pretreatment in this study did not need complex process of detoxification. After neutralization of the acid-autoclaved pretreated waste with NaHCO_3 , the mixture can be hydrolysed further by Cellic[®] CTec2 and fermented by instant dry bakers yeast (*S. cerevisiae*) for ethanol production. The ethanol yield was investigated between sterilization and non-sterilization of the hydrolysate before yeast inoculation and different concentration of yeast. After acid-autoclaved pretreatment, enzymatic hydrolysis is used to convert the residual cellulose into monomeric sugars. The sugar is then fermented to ethanol by yeast. When enzymatic hydrolysis and fermentation are performed sequentially, it is referred to as separate hydrolysis and fermentation (SHF). However, the two process steps can be performed simultaneously, i.e. simultaneous saccharification and fermentation (SSF) (Öhgren et al., 2007).

In this study, the fermentation of non-sterile hydrolysate is similar to SSF with the delayed time of yeast inoculation and the sterile hydrolysate is similar to SHF owing to the denaturation of enzyme by autoclave. As can be seen from Figure 4, the non-sterile hydrolysate gave more yield of ethanol than the sterilized one (264.96 ± 0.51 and 159.96 ± 0.74 mg/g dry wt, respectively). During SSF, the faster saccharification rates result because the glucose product is immediately removed, considerably diminishing its inhibitory effect on the cellulase system (Abe & Takagi, 1991). Furthermore, small amount of fermentable sugar continuously gradually released by the enzyme, as can be seen from the stable low amount of sugar after 2-3 days of fermentation in Figure 4 & 5.

Rattanachomsri et al. (2009) found that the enzyme saccharification reaction can be performed simultaneously with the ethanol fermentation process using a thermotolerant yeast *Candida tropicalis* BCC7755. This process produced 14.3 g/l ethanol from 4% (w/v) cassava pulp after 30 h of fermentation. The productivity rate of 0.48 g/l/h is equivalent to 93.7% of the theoretical yield based on total starch and cellulose, or 85.4% based on total fermentable sugars. Öhgren et al. (2007) has compared the yield of ethanol from steam-pretreated corn stover by two different process of fermentation, SSF and SHF, using 8% water-insoluble solids. They reported that SSF gave a 13% higher overall ethanol yield than SHF (72.4% versus 59.1% of the theoretical). In addition, Apiwatanapiwat et al. (2013) also found that the ethanol concentration produced from cassava pulp using the SSF process was higher than that using the SHF process. Combination of the two process steps also results in a lower capital cost, the reduction of the requirements for many reactors and the fact that the ethanol concentration is higher during SSF than SHF reduces the risk of contamination (Adekunle et al., 2016). Zhang et al. (2013) found that the simultaneous co-saccharification of cassava starch/cellulose and ethanol fermentation process provided a cost effective option of cassava cellulose utilization for ethanol production. However, the temperature optima for the yeast and the enzymes used differ, which means that the conditions used in SSF cannot be optimal for both the enzymes and the yeast (Öhgren et al., 2007).

Furthermore, increase the hydrolysis time and the size of yeast inoculum provided more ethanol, as shown in Figure 4 and 5. Fermentable sugars should be as high as possible in the bioethanol fermentation system to reduce the cost (de Albuquerque Wanderley et al, 2013). The increase in sugar concentration up to a certain level caused fermentation rate to increase. The initial sugar concentration also has been considered as an important factor in ethanol production. High ethanol productivity and yield in batch fermentation can be obtained by using higher initial sugar concentration. Fermentation time also affect the growth of microorganisms. Shorter fermentation time causes inefficient fermentation due to inadequate growth of microorganisms. On the other hand, longer fermentation time gives toxic effect on microbial

growth especially in batch mode due to the high concentration of ethanol in the fermented broth (Zabed et al., 2014).

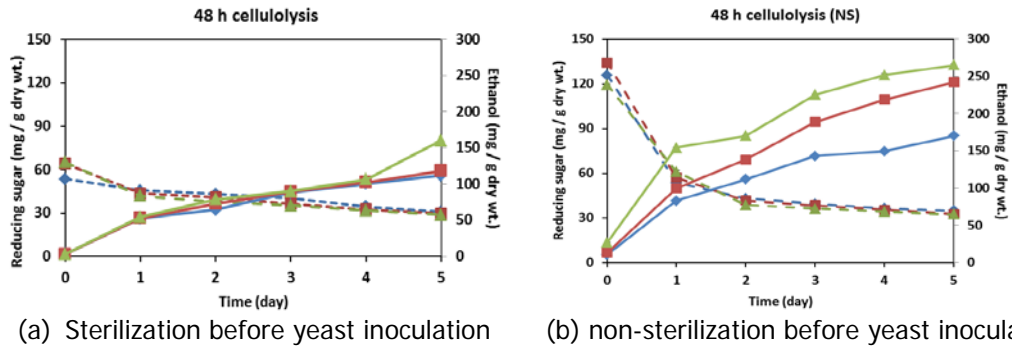


Figure 4. Effect of substrate sterilization and yeast concentration (3%, 6% and 9% (w/v)) on ethanol production, using 1% (v/v) acetic acid pretreated cassava waste with 6% (w/w) Cellic® CTec2 at 50°C for 48 h as substrate, fermented for 5 days at room temperature. (dash line for sugar, solid line for ethanol; ◆ = 3% yeast, ■ = 6% yeast, ▲ = 9% yeast)

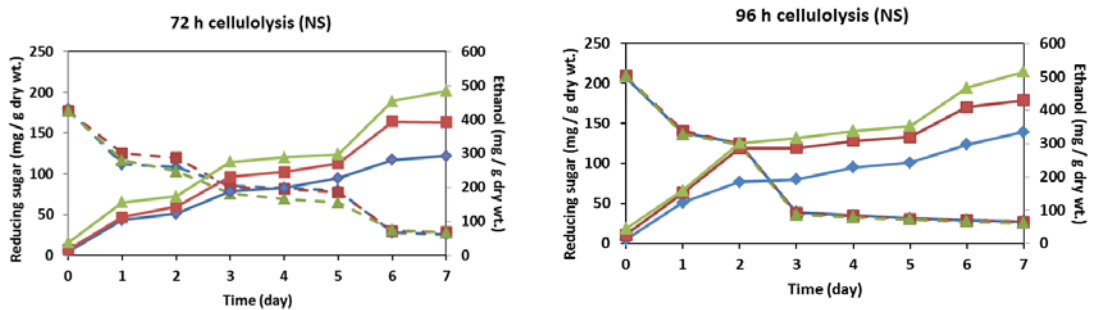


Figure 5. Effect of cellolytic time of cassava waste (72 h and 96 h) on ethanol production, using various concentration of yeast (3%, 6% and 9% (w/v)) fermented for 7 days at room temperature without substrate sterilization before yeast inoculation. (dash line for sugar, solid line for ethanol; ◆ = 3% yeast, ■ = 6% yeast, ▲ = 9% yeast)

Inoculum concentration may give significant effects on the ethanol production. Duhan et al., (2013) found that when the inoculum size was increased from 5 to 10%, ethanol production was also increased but above 10%, rate of alcohol production decreased after 48 h of incubation. The production of ethanol was increased with the increase in cell numbers from 1×10^4 to 1×10^7 cells per ml but there was no significant ethanol production found between 10^7 and 10^8 cells per ml. This is because the increase in cell concentration within certain range reduces fermentation time as the cells grow rapidly and directly consumes sugars into ethanol (Zabed et al., 2014). Breisha (2010) reported that increasing the yeast inoculum volume from 3% to 6% showed positive effects on fermentation from 25% sucrose and reduced the fermentation time from 72 h (3%) to 48 h (6%). The fermentation time shorten along with the raise in inoculum size which was due to the fast cell growth within the reactor. Most of the substrate was immediately converted to ethanol. The common inoculum size employed in bioethanol production is 5% and 10% (Azhar et al., 2017).

Conclusion

This study shows that cassava cellulosic waste obtained from starch processing can be utilized for the production of bioethanol. The actions of acetic acid pretreatment and commercial cellulase have a remarkable capacity to degrade this waste. Pretreatment of the waste with 1% (v/v) acetic acid in the autoclave, followed by hydrolysis with cellulase at conditions of pH 5.5, temperature 50 °C, time 96 h produces more reducing sugar. The yield of 515.39 ± 0.48 mg ethanol/g dry wt was obtained from 9% (w/v) of *Saccharomyces cerevisiae* in 7-days fermentation at room temperature. Further study will be the application of this pretreatment method with other types of waste to see how well it works.

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