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Enzymatic hydrolysis of cassava chips for high gravity ethanol fermentation

Suthkamol Suttikul[1,*], Jutamas Panthong[1], Vishnu Panphan[1] and Thapparait Kanhanont[1]

[1]Expert Center of Innovative Clean Energy and Environmental, Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand
[*]Corresponding author: suthkamol@tistr.or.th

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Abstract

Very high gravity (VHG) ethanol fermentation has been developed to enhance productivity via higher ethanol concentration. We investigated simultaneous saccharification and fermentation (SSF) of cassava chips under high gravity ethanol fermentation. Cassava chips were ground into small granules and dissolved in water to reach 30% (w/w) of dry solid. The viscosity of the mash was minimized by pretreatment with a viscosity reduction enzyme. The starch in the pretreated mash was liquefied to maltodextrins by the action of thermo-stable α-amylase (0.1%, w/w) at 85 ˚C for 90 min. SSF of liquefied mash was performed at 35 ˚C with the simultaneous addition of glucoamylase, thermotolerant yeast (*Saccharomyces cerevisiae*), and 0.5 g/L of urea as a nitrogen source for the yeast. The optimal glucoamylase loading was 0.6% (w/w). Under these conditions, the SSF process was completed after 60 h. The ethanol content achieved at 117.17±1.44 g/L or 14.85% (v/v), corresponding to 85.56% of the theoretical ethanol yield.

Keywords: High gravity fermentation, Liquefaction, Simultaneous saccharification and fermentation, Ethanol production

1. Introduction

Due to the increasing price of oil, bioethanol is regarded as an ideal candidate to replace the role of fossil fuel. Thus, research on this renewable source has become increasingly important to humans, especially in terms of improving productivity and efficiency, and decreasing production costs. In Southeast Asia, cassava has been considered an attractive raw material for bioethanol production [1].

Very high gravity (VHG) fermentation technology can considerably increase both fermentation productivity and ethanol concentration while consuming less water and energy [2,3]. The application of high gravity (HG) and VHG fermentation technologies (the preparation and fermentation of mashes containing 20-26 g (HG) and ≥27 g (VHG) dissolved solids per 100 g mash) have been proposed for efficient ethanol production and to reduce stillage volume [4,6]. Among different ethanol production methods, the HG and VHG fermentation technologies are very efficient at the industrial scale since they offer higher ethanol yield (11-14% for HG and 15-18% for VHG), low waste generation, and low operation costs. Higher ethanol levels can reduce the distillation cost compared to traditional fermentation, where a maximum of 7-10% ethanol is produced [4-6].

Despite HG or VHG ethanol fermentation having several advantages, it causes the fermentation process to become stuck, or sluggish, when yeast cells are exposed to several stresses. The high concentration of dissolved solids increases external osmotic pressure, and ethanol concentration can be raised to the toxic level for yeast cells during HG or VHG fermentations. Osmotic and ethanol stresses result in a loss of cell-viability, growth, and yeast fermentation performance [7]. These often lead to a prolonged fermentation period and an increase in the amount of unfermentable sugar, consequently, the efficiency of ethanol production decreases. The essential background of yeast strain on stress tolerance, including osmotic pressure, temperature, and ethanol, is, therefore very important to overcome this challenge. The simultaneous saccharification and fermentation (SSF) process is preferable in industrial applications due to its high ethanol yield, low energy consumption, and short processing time. In the SSF process, saccharification is performed together with ethanol fermentation, instead of as a separate

step after hydrolysis. Thus, the high carbohydrate content inhibition of the starch hydrolysis is avoided because the fermenting yeast immediately consumes the released sugars. Today, many commercial fuel ethanol production facilities employ VHG fermentation, which increases the volumetric productivity and cost-effectiveness of the SSF process [8]. A previous study on ethanol production from fresh cassava mash showed that high viscosity caused resistance to solid-liquid separation and lower fermentation efficiency [9]. High viscosity causes several handling difficulties during processes and may lead to incomplete hydrolysis of starch to fermentable sugar [10,11]. However, suitable mash viscosity can be accomplished via enzymatic hydrolysis of the cell-wall matrix, with cellulase and pectinase reported to reduce the mash viscosity of agricultural residues from sweet potato [7], sugar beet [12] and cassava [13].

This research aimed to determine the optimum condition of the liquefaction step and the effect of viscosity reduction enzyme and glucoamylase dosage on ethanol production under HG conditions from cassava chips.

2. Materials and methods

2.1 Raw material

Cassava chips were obtained from Kamphang-Phet province, Thailand. They were milled into small granules (flour) and stored at room temperature for the experiments.

2.2 Enzyme and yeast preparation

The commercial enzymes used in this study were a viscosity reduction enzyme, Viscozyme® cassava R (100 Fungal Beta-Glucanase Unit /g), a thermostable-α-amylase, Liquozyme® SC DS (240 Kilo Novo alpha-amylase Unit /g), and glucoamylase, Spirizyme® fuel (750 Amyloglucosidase Unit /g) from Novozymes. Active dry yeast, *Saccharomyces cerevisiae*, Thermosacc® DRY (Lellamand, France) was used throughout the study. According to the producer's data, the yeast contained 2×10^{10} of viable cells/g. Prior to inoculation, 0.036 g of yeast was rehydrated in 100 mL of sterile distilled water for 15-30 min with regular agitation, following the recommended dosage and procedure of the dry yeast manufacturer. Urea was used as a nitrogen supplement.

2.3 Optimization of liquefaction

Cassava chip slurry was made with 30% w/w dry solid (DS). A slurry was macerated with 0.05% (w/w) of viscosity reduction enzyme, containing the activity of β-glucanase, Xylanase, Cellulase, and Hemicellulase. The slurry was incubated with stirring at 50 ˚C for 30 min, following the recommended method of the enzyme manufacturer, then liquefied with a heat-stable-α-amylase at different enzyme dosage (0.05, 0.1, and 0.15% (w/w) of dry solid). The slurry was instantaneously heated to 85 ˚C or 95 ˚C and held at this temperature for 180 min. Aliquot samples were collected every 30 min. To determine the liberated reducing sugars, the enzyme reaction was stopped by the addition of 0.4 mM $HgCl_2$ [7]. The samples were centrifuged at 9,000 rpm for 10 min. The liquid fractions were then used for determining dextrose equivalent (DE), which indicates the ratio of reducing sugar (quantified by the Nelson-Somogyi method) and total sugar (analyzed be Phenol-Sulfuric acid method) [7].

DE was determined using the following equation;

$$\%\, DE = \frac{Reducing\ sugar\ (g/L)}{Total\ sugar\ (g/L)} \times 100 \quad (1)$$

2.4 Effect of viscosity reduction enzyme on ethanol production in 5L fermenter

The cassava chip slurry of 30% (w/w) dry solid matter was hydrolyzed with and without the addition of 0.05% (w/w) of the viscosity reduction enzyme at 50 ˚C for 30 min. The slurry was then liquefied with a heat-stable-α-amylase at optimum dosage, temperature, and incubation time. The liquefied mash was cooled to 35 ˚C and simultaneously added with 0.45% (w/w) of glucoamylase, 0.5 g/L of urea, and yeast inoculum. The SSF process was completed in 5 L fermenter at a 50-100 rpm agitation speed, without aeration. Samples were periodically withdrawn and used for the analysis of reducing sugar, cell number, and ethanol, in order to compare the ethanol yield.

2.5 Effect of glucoamylase dosages on SSF of cassava chip

The SSF experiments were carried out in 5 L fermenter, containing 30% (w/w) of cassava chip mash as a carbon source. After the cassava chips were processed via viscosity reduction and liquefaction steps, the liquefied mash was cooled to 35 ˚C. The glucoamylase at different dosages (0.30, 0.45, and 0.60% (w/w) were then simultaneously added into the mash with 0.5 g/L of urea and yeast inoculum. The SSF process was completed at a 50-100 rpm agitation speed, without aeration. The samples were periodically withdrawn every 12 h and used for the analysis of reducing sugar, cell number, and ethanol, to evaluate the effects of glucoamylase dosage on ethanol yield.

2.*6 Analytical methods*

The protein, fat, ash, and fiber content of the cassava chips were analyzed using the AOAC method [14]. The composition of starch was determined using the enzymatic glucoamylase AACC method [15]. The total and reducing sugar were analyzed by the Phenol-sulfuric acid method and the Nelson-Somogyi method, respectively [16-17]. The cell number was determined using a haemacytometer. Ethanol concentration was analyzed using gas chromatography, Agilent 6890 series (Agilent GC system, USA) with 19091N-133 Innowax column and flame ionization detector.

3. Results and discussion

3.1 Chemical composition of raw material

The composition of the cassava chips was analyzed and is summarized in Table 1. The moisture content of the cassava chips was 11.09%. The cassava chips were comprised of 71.22% starch, 1.83% protein, 0.17% Fat, 4.94% ash, and 6.96% fiber on dry solid basis. The results showed that starch was the main composition of these raw materials, which have the potential for ethanol production.

Table 1 The composition of cassava chips.

Chemical composition	% Dry weight
Protein	1.83±0.02
Fat	0.17±0.01
Ash	4.94±0.00
Fiber	6.96±0.01
Starch	71.22±0.24

The data presented are mean for triplicate assays.

3.2 Optimization of liquefaction

The liquefaction step aimed to convert the starch of the cassava chips into maltodextrins at high temperature using thermo-stable-α-amylases [1]. The high temperature for starch gelatinization was necessary to increase the enzyme digestibility of cassava starch because raw cassava starch is resistant to enzyme hydrolysis [18]. Dextrose equivalent (DE) was measured as an indicator of liquefaction [7]. Figure 1 demonstrates that using 30% w/w (DS) of cassava chips increases DE values according to the enzyme dosage. When using 0.05% (w/w) α-amylase at 85 ˚C and 95 ˚C, the DE values were significantly lower than using an enzyme dosage of 0.1 and 0.15% (w/w). The DE values at 85 ˚C were observed to be similar to those obtained at 95 ˚C when using an enzyme dosage of 0.1 and 0.15% w/w (DS) after 90 min of incubation. Thus, a liquefaction temperature of 85 ˚C would be sufficient for complete starch gelatinization. In order to save energy cost, the heating temperature of 85 ˚C was selected. Therefore, in this study, the optimum conditions for liquefaction were 0.1% w/w (DS) of α-amylase, a heating temperature of 85 ˚C, and incubation time of 90 min. These conditions resulted in a liquefied mash with a DE of 25.81±0.13. In other work, Srichuwong et al. (2009) used a similar temperature (85 ˚C) for the liquefaction step of potato mash [7]. Nguyen et al. (2014) used a lower temperature (only 80 ˚C) for the liquefaction step, the energy was accordingly saved [1], while Yingling et al. (2011) used a gelatinization step with different enzyme doses, followed by autoclaving at 121 ˚C for 15 min, to completely breakdown the starch [8].

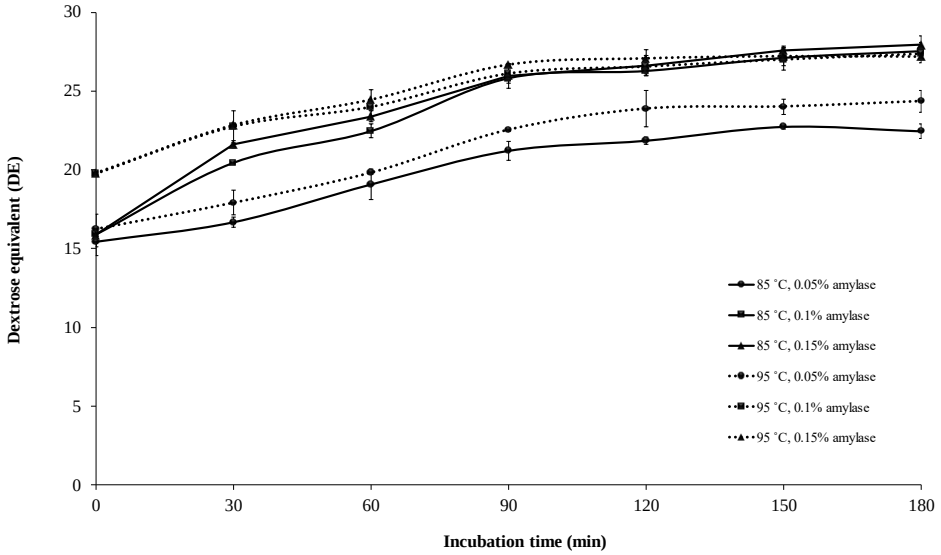

Figure 1 Dextrose equivalent (DE) obtained from the liquefaction of cassava chips at 85 ˚C and 95 ˚C with different enzyme dosages.

3.3 Effect of viscosity reduction enzyme on ethanol production

In HG or VHG processes, cassava slurry viscosity during liquefaction plays an important role, which can decrease enzyme efficiency in starch hydrolysis, thus reducing the ethanol yield. Therefore, decreasing starch-paste viscosity is a prerequisite for conducting ethanol production at HG or VHG. Different methods have been previously used to resolve this problem. Typically, the viscosity reduction enzymes such as cellulase, hemicellulase, pectinase [7], or xylanase [19] were added into the mash to reduce the mash viscous. In this study, the effect of a viscosity reduction enzyme on ethanol production was demonstrated in Figure 2. The results showed that a similar ethanol concentration was obtained either with or without the addition of a viscosity reduction enzyme prior to the liquefaction step and SSF process. The results imply that using the viscosity reduction enzyme does not significantly promote ethanol production. As seen after 48 h, ethanol concentration in processes with and without the viscosity reduction enzyme were 102.62 and 99.11 g/L, respectively. However, a viscosity reduction enzyme has benefits in industrial production. This is because in an ethanol production factory, the liquefied mash is generally transferred to the fermenter via pipeline and the viscosity reduction enzyme can facilitate the flow. In order to deliver the results to the industrial ethanol production scale, the viscosity reduction enzyme would be used in further experiments. Srichuwong et al. (2009) studied the VHG process of sweet potato at 28% dry matter. The enzymatic hydrolysis with the cell-wall degrading enzymes (cellulase, hemicellulose, and pectinase) was conducted at a similar temperature (50 ˚C for 50 min) used in this study [7].

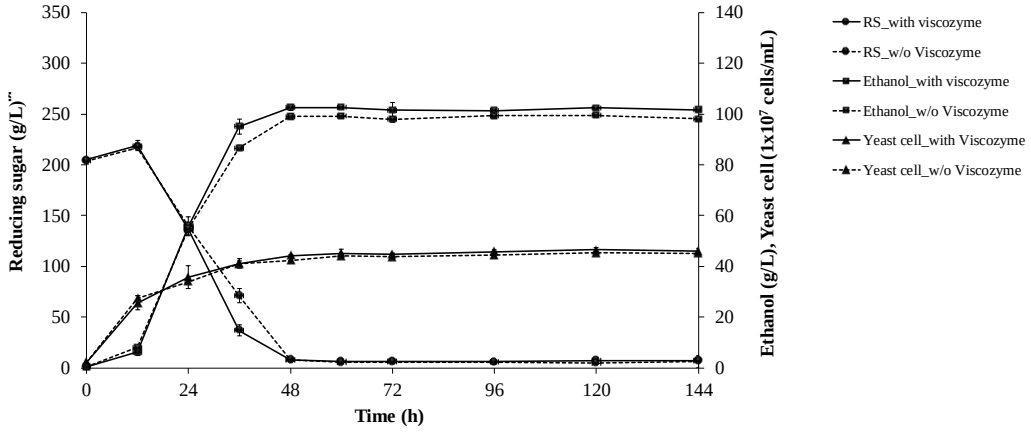

Figure 2 Time course of simultaneous saccharification and fermentation (SSF) of high gravity (HG) cassava chip mash with and without the addition of the viscosity reduction enzyme.

3.4 Optimization of SSF

Ethanol fermentation was performed using the SSF process, where glucoamylase, nitrogen source, and yeast, were simultaneously added. SSF process decreases the product inhibition of an enzyme and the osmotic stress on the yeast cells. In this study, SSF was operated at 35 ˚C, which was lower than the optimum temperature of the added saccharifying enzymes (Spirizyme fuel, 50-60 ˚C). Urea was used to provide assimilable nitrogen for the yeast in the fermenter as previous studies have shown that urea can improve the growth of yeast cells and increase ethanol production [20,21]. The effects of enzyme dosage and fermentation time on ethanol yield were investigated. The results showed that ethanol concentration obtained at an enzyme dosage of 0.30, 0.45, and 0.60% (w/w) were 94.64±1.10, 105.28±0.41, and 117.17±1.44 g/L, respectively (Figure 3). Extending the fermentation time from 60 to 96 h had no statistically enhancing effect on ethanol yield. Thus, the optimum fermentation time was 60 h, and the optimum glucoamylase dosage was 0.60% (w/w), which gave a maximum ethanol concentration of 117.17±1.44 g/L or 14.85% (v/v), corresponding to 85.56% of the theoretical ethanol yield. The HG technology resulted in a higher ethanol production (11-14%, v/v) compared with the 7-10%, v/v from a conventional process [4-6]. In addition, the SSF process of this study was operated at 35 ˚C, the energy for cooling fermenter can then be saved. In comparison, Puligundla et al. (2014) developed VHG ethanol production from 30% cassava mashes at 30 ˚C, achieving an ethanol concentration of 12.8% (v/v) with a fermentation efficiency of 75.8% [22]. Nguyen et al. (2014) operated SSF process of 31.5% (w/v) cassava flour at 30 ˚C, the ethanol titer achieved 17.2% (v/v), corresponding to 86.1% of the theoretical ethanol yield at lab scale [1].

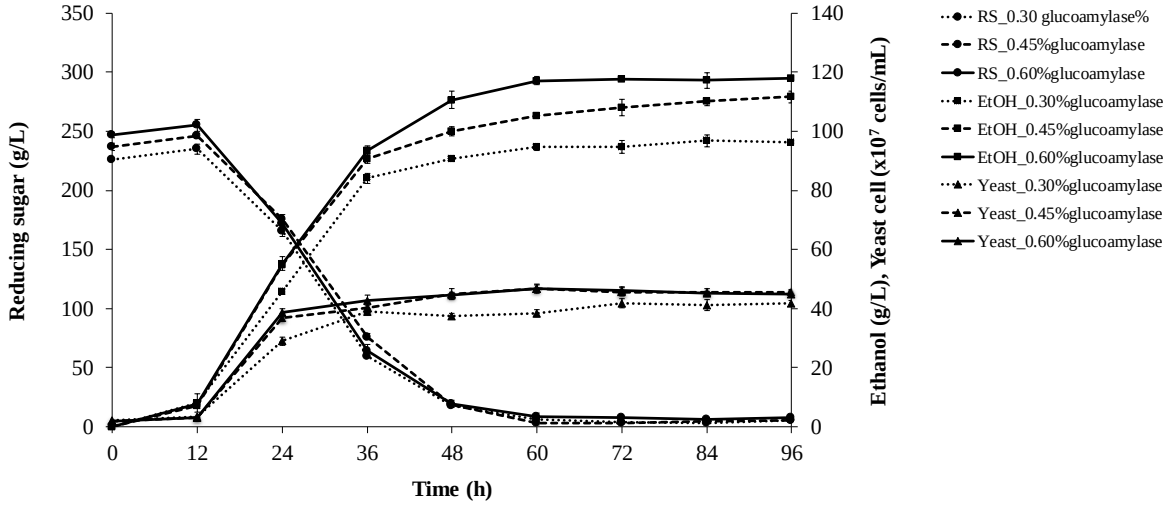

Figure 3 Time course of simultaneous saccharification and fermentation (SSF) of high gravity (HG) cassava chip mash at different glucoamylase dosage.

4. Conclusion

Thermo-stable-α-amylase and viscosity reduction enzymes were applied to hydrolyze and reduce the viscosity of cassava chip mash, to improve process handling, and stimulate the HG condition for subsequent ethanol fermentation. At the first stage, 30%w/w dry solid of cassava chip was hydrolyzed with 0.05% (w/w) of viscosity reduction enzyme at 50 ˚C for 30 min. The slurry was then liquefied with 0.1% (w/w) of alpha-amylase at 85 ˚C for 90 min. Ethanol fermentation was accomplished by the SSF process at 35 ˚C, where 0.6% (w/w) of glucoamylase, 0.5 g/L of urea, and active dry yeast were simultaneously added. After 60 h, an ethanol concentration of 117.17±1.44 g/L or 14.85% (v/v) was obtained, which was equivalent to 85.56% of the theoretical ethanol yield. The efficient HG- SSF process described in this study would benefit the starch-based ethanol industries without alteration of plant equipment.

5. Acknowledgment

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