Application of ultrasound and technical enzymes during bioethanol production from fresh cassava root

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Abstract

The effect of pre-treatment on sugar yield of cassava root as well as application of amylolytic enzymes for conversion of starch to glucose and bioethanol production using yeast were studied. Different pre-treatments, such as ultrasound, sulphuric acid as well as application of technical enzymes (pectolytic/cellulolytic), have effect on starch conversion into glucose. Up to 3% or 8% of free sugar could be observed after ultrasound and technical enzyme treatment, respectively, compared to untreated sample (free sugar 0.5%). Amylolytic enzymes (α-amylase and gluco-amylase) from different companies (Novozymes, Stern, Valley and AB enzymes) investigated in this study were effective to convert starch in glucose. The activity of amylolytic enzymes depended on the concentration of enzymes and treatment time. Up to 100% of starch could be converted in glucose.

Introduction

In recent years, the fuel ethanol industry in the world has grown rapidly, spurred by renewable energy concerns, new fuel ethanol standards and government incentives. Fuel ethanol is produced mostly from corn starch, but cassava, a perennial woody shrub with up to 32% (fresh) starch content, has attracted more and more attention recently. As feedstock for fuel ethanol, cassava has two advantages over other feed stocks. First, cassava can be cultivated on marginal lands where other crops, such as corn, wheat, rice and sugarcane cannot be grown well. Second, cassava is not a staple food for some country such as the Thai or Chinese people. Using cassava for ethanol production would not raise major ethical and moral issues as would corn ethanol.

Pectolytic enzymes are used to catalyze the hydrolysis of pectin materials. Complete hydrolysis leads to the production of pectic acids. Pectic substances are found widely in plant tissues, particularly in fruits. While pectic enzymes occur in many fruits, the commercial enzyme products are derived from strains of the fungus Aspergillus niger. The commercial enzymes are mixtures of several pectic enzymes, particularly pectin methylesterases and polygalacturonases. Differences of the commercial preparations are due to variations in the kinds and amounts of particular pectic enzymes present. Enzymatic saccharification takes place in three stages, a pre-liquefaction cooking and then liquefaction both with α-amylase, followed by pre-saccharification with glucoamylase, and then simultaneous saccharification and fermentation. Combined saccharification and fermentation requires 72 h.

Ultrasound waves are similar to sound waves but have a frequency above 16 kHz and cannot be detected by the human ear. When ultrasonic waves hit the surface of a material, a force is generated. If the force is perpendicular to the surface, it results in compression wave through the food, whereas if the force is parallel to the surface it produces a shearing wave. Ultrasound produces very rapid localized changes in pressure and temperature that cause shear disruption, cavitations (creation of bubbles in liquid foods) thinning of cell membranes, localized heating and free radical production, which have a lethal effect on microorganisms.

The aim of this research was to study the effect of pre-treatment on sugar yield of cassava root as well as application of amylolytic enzymes for conversion of starch to glucose and bioethanol production using yeast.

Material and Equipment

Fresh cassava root from province Nakhornpathom (origin Rangsit) was used in this study. Cassava root were washed with tap water and the peel (inner layer and outer layer) was removed. Peeled root were put in plastic bag and stored at -20°C. The prepared root were thawed and cut in strips of 20 to 30 mm length and 2 mm edge length using a kitchen blender. Technical enzymes used in this project were pectinase and cellulase (from Company Valley, USA; AB Enzyme, Germany; and Novozymes, Denmark), α-amylase, gluco-amyrase (from Company Stern Enzyme, Germany, Novozymes, Denmark; Valley, USA, Dr. Luca, Germany and AB Enzyme, Germany). Saccharomyces cerevisiae yeast from company AB-Enzyme (Germany) was used for fermentation of saccharified...
cassava root to bioethanol.

Ultrasound equipment used in this study (Sonopulse type 302, Bandelin, Germany) had the maximal power of 200 W and power density of max. 150.38 W/cm² (diameter of Sonotrode tip 1.3 cm).

Pretreatments

Enzyme treatment using pectolytic/cellulolytic enzymes: A sample of 200 g of ground cassava was mixed with 0.5% w/w of enzyme mixture including 0.5 g Crystalsyme + 0.5 g Validassum ANCL (Company Valley USA) or 1 g of enzyme mixture including 0.33 g Rohapect Max + 0.33 g Rohulase CL + 0.34 g Rohapect D5+special (AB Enzyme, Germany) or 0.5 g cellulase + 0.5 g xylolase (Dr. Luca and Partner Ingenieurkontor Gmbh, Berlin, Germany), respectively. The pH of enzyme mixture and cassava was adjusted to pH 4.0 using 2 M HCl. The enzyme treatment took place at 40°C in water bath for 3 h.

Treatment of sample using sulphuric acid: Each of 200 g of ground sample was mixed with 100 ml of 1 M, 2 M and 4 M sulphuric acid. The beakers were heated in water bath at 95°C for 2 h. After acid treatment the samples were cooled at room temperature and neutralized with 10 M NaOH solution to pH 7±0.5.

Treatment of sample using ultrasound: Of ground cassava 200 g was placed in a beaker and treated with ultrasound at 50% power (100 W) for 20 and 30 min. The temperature increase during ultrasound treatment was less than 10°C (maximum temperature of sample after ultrasound treatment was 40±5°C). After ultrasound treatment the samples were cooled up to room temperature.

Sacharification using amylolytic enzymes: For converting of starch in glucose (sacharification) α-amylase and gluco-amylase from different companies (Valley company, USA; Novozymes, Denmark; Stern Enzyme GmbH & Co. KG, Germany; AB Enzymes GmbH, Germany) were used. To find the optimal concentration of enzyme the amount of enzymes varied between 0.025% w/w to 0.1% w/w of sample.

Of ground cassava 200 g was placed in a 400 ml beaker and α-amylase added. Then the pH of mixture was adjusted to optimal pH of α-amylase from different companies using 1 M CaO solution. The mixture of cassava and enzyme were heated in water bath up to 80°C for 1 h. During heating the mixture was mixed intensively for better heat transfer and mixing the enzymes. After treatment with α-amylase, the sample was cooled in water bath up to 50°C. For saccharification gluco-amylase (from different companies) was added in cooled sample (50°C) and adjusted pH up to 4.2±0.2. The sample was incubated in a water bath at 50°C for 3 h. After each hour a small amount of sample (1 g) was taken out to measure glucose content and determine the kinetic of glucose production during enzyme treatment.

Preparing of sample for fermentation experiment

Fermentation: Of ground cassava root strip 3 kg was added in a stainless steel pot and adjusted to pH 6.3 using CaO solution (10%). Then 0.1% w/w of α-amylase enzyme (from company Valley) was added into the sample and the sample heated on a heat plate with vigorous mixing for 100 min at 80°C. During this step starch in cassava was thermally gelatinized. The viscosity of gelatinized starch was reduced rapidly due to α-amylase activity. After α-amylase treatment, the sample was cooled up to 50°C and adjusted at pH 4.5 using 2 N HCl. Distilled water was added to achieve the initial weight of sample (3 kg). For saccharification 0.1% w/w gluco-amylase (Valley company) was added in the sample and incubated in water bath for 4 h with occasionally stirring. Then the saccharified sample was filtered through 2-layer cheese cloth to produce pulp free juice.

The filtrate was divided in two parts. One part was adjusted at pH 4.0 and another part to pH 5 using 2 N HCl. Each sample (250 ml) was poured in 3 conical flasks (each flask approximately 250 ml) and covered with cotton. The samples were sterilized in an autoclave at 121°C for 15 min. After autoclaving the samples were cooled at room temperature and inoculated with approximately 2.5 g S. cerevisiae yeast granular (AB enzymes, Germany) at sterile condition in clean bench. For investigation of temperature effect on fermentation time, flasks were fixed on shaker with 150 rpm at 30°C. Fermentation at 37°C was carried out without shaking. After each day one sample (approximately 20 to 30 g) was taken out from flask under sterile condition in a clean bench and used for determination of sugar residue, ethanol content as well as total count of yeast.

Analytical methods: Measurement of free reducing sugar in raw material as glucose was carried out using method of International Starch Institute Science Park (Aarhus, Denmark).

Measurement of starch after saccharification of starch into glucose: Five g of fresh material was weighed in a 100 ml conical flask containing 25 ml of 80% ethanol. The flask was left overnight and filtered using Whatmann No.1 filter paper. The residue was washed with distilled water twice and transferred proportionally into a 100 ml conical flask. 20 ml 2 N HCl was added and hydrolysed by heating for 20 min in a water bath at 90°C. The completion of hydrolysis was measured by the absence of blue colour with N/10 iodine solution. The hydrolysed solution was diluted to 100 ml and after further dilution to obtain approximately 100 ppm, the glucose was estimated by Luff-Schoorl method for sugar estimation.

Measurement of water content: Five g of fine grinded sample was put in a pre-dried and cooled glass dish and dried at 103±5°C for 2 h in a hot air oven. After drying the sample was cooled in desiccator and weighed again. The water content was determined gravimetrically.

Measurement of fibre: The sample was oven-dried at 105°C. Then 2 g of dried cassava root was placed in a beaker and 200 ml of boiling 1.25% H₂SO₄ was added. The mixture was heated for 30 min. After cooling the mixture was filtered on a filter paper and the residue was washed with 25 ml of 96% alcohol. The residue was heated at 600°C for 30 min. The fiber content was calculated using following equation:

\[
\text{% Crude fiber in the ground sample} = \frac{\text{(loss in weight on ignition)-10}}{\text{weight of sample}}
\]

Measurement of ash: Ten g of dried cassava root was put in a pre-weighed dried crucible. The sample was burned in a lamp until the
colour of sample was grey. The ashed sample was cooled in a desiccator and weighed again. Ash content was calculated as follows:

\[
\text{Ash content on dry basis} = \frac{\text{weight of sample after burning}}{\text{weight of sample before burning}} - 100
\]

Measurement of fat: Fat content was determined by Soxhlet method after 6 h extraction of the sample using boiling hexane. After evaporation of hexane the fat content was determined gravimetrically.

Measurement of ethanol content: Two methods were used for measurement of ethanol:

A. Refractometric method: The refractometer value of different ethanol solutions with defined ethanol content (as w/v of water/ethanol) was measured and a standard curve was drawn. These standard curves were used for measurement of unknown ethanol content in fermented cassava. It was important that the sample must be free from dissolved sugars and other soluble solid materials. The distillation of sample before ethanol measurement ensured that only ethanol can be measured during refractometric measurement.

B. Gas chromatographic method: The ethanol measurement was carried out after AOAC Official Method 983.14 using gas chromatography method. A standard curve for ethanol measurement using GC method was determined.

Enumeration of survivors: Before and after each treatment viable counts of the surviving yeasts were determined by standard plating technique. Every sample was serially diluted in sterile 0.5% saline solution (dilution 1:10), plated in Petri dishes containing potato dextrose agar and incubated at 30°C for 3 days before counting. Each experiment was carried out three times at least and the arithmetic mean was reported as final result.

Results

Composition of raw material: Sugar, starch, water, fiber, ash and fat content in fresh cassava are shown in Table 1.

<table>
<thead>
<tr>
<th>Composition</th>
<th>g/100 g fresh cassava</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch</td>
<td>24.80</td>
</tr>
<tr>
<td>water</td>
<td>62.60</td>
</tr>
<tr>
<td>ash</td>
<td>1.69</td>
</tr>
<tr>
<td>fiber</td>
<td>0.61</td>
</tr>
<tr>
<td>fat</td>
<td>0.38</td>
</tr>
<tr>
<td>sugar</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 1. Composition of fresh cassava root.

Preparing standard curves for ethanol
As shown in Fig. 1, a good correlation existed between measured refraction index and ethanol concentration in model solution. The gas chromatography (GC) method used in this study was suitable for ethanol measurement as well. A good correlation between ethanol content in standard solution and measured ethanol was found using GC method (Fig. 2).

Effect of pretreatments on free sugar content in cassava root
Pretreatment using acid combined with heating: Pretreatment of cassava samples with 1, 2 and 4 molar sulphuric acid (2 part cassava to 1 part acid) at 90°C for 2 h treatment time resulted conversion of starch to glucose (Table 2). With increasing the concentration of acid, the sugar content decreased from 24 to 13.5%. This is maybe because of degradation of glucose to hydroxyl methyl furfural (HMF) and polymerization reaction to black brown sugar color at high acid concentration and temperature. From this data the conversion of starch to glucose should be carried out using diluted sulphuric acid with concentration of ≤ 1 molar to minimize the degradation of glucose to HMF.

<table>
<thead>
<tr>
<th>Concentration of sulfuric acid</th>
<th>Sugar (g/100g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 molar</td>
<td>23.97</td>
</tr>
<tr>
<td>2 molar</td>
<td>17.48</td>
</tr>
<tr>
<td>4 molar</td>
<td>13.55</td>
</tr>
</tbody>
</table>

Table 2. Percent free sugar after acid hydrolysis of fresh cassava root.

Pretreatment using pectolytic/cellulolytic enzymes: Pretreatment of fresh cassava using pectolytic/cellulolytic enzymes increased the free sugar from 0.5% in raw material up to 3.28, 3.48 and 8.1% after treatment with Dr. Luca; AB enzyme and Valley enzyme, respectively (Table 3). This showed that the cell wall biomaterial (pulp) such as cellulose/hemisellulose could be digested to glucose in presence of pectolytic/cellulolytic enzymes. The main compounds of cellulose/hemicellulose consist of glucose substance that could be free after enzyme treatment.

<table>
<thead>
<tr>
<th>Enzyme type</th>
<th>Sugar content (g/100g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Luca</td>
<td>8.02</td>
</tr>
<tr>
<td>AB enzyme</td>
<td>3.48</td>
</tr>
<tr>
<td>Valley enzyme</td>
<td>3.28</td>
</tr>
</tbody>
</table>

Table 3. Sugar content after enzyme treatment using pectolytic/cellulolytic enzymes.
Pretreatment using ultrasound: Using high power ultrasound, the amount of total reducing sugar in fresh cassava increased (Table 4) from 0.5% in untreated sample up to 2.37 and 3.03% after 20 and 30 min of ultrasound treatment, respectively. It is not clear from this experiment, if the increasing of sugar content is because of strong shear force and cavitation effect during ultrasound treatment causing degradation of high molecular substances such as cellulose/hemicellulose and starch or because of simultaneous effect of plant native enzymes and ultrasound effect.

Saccharification of cassava root: To determine the effectiveness of amylolytic enzymes (α-amylase and gluco-amylase) supplied from different companies, conversion of starch to glucose using amylolytic enzymes from companies, Stern (Germany), and Novozymes (Denmark) at concentrations of 0.05 and 0.1% w/w were studied. Increasing the enzyme concentration from 0.05 to 0.1% w/w increased the conversion ratio of starch to glucose in the case of Stern enzymes as well as Novozymes (Figs 3 and 4). In general, the Novozymes enzyme showed higher effect than Stern enzymes. Up to 95% of starch could be converted in glucose after 3 h enzyme treatment using α-amylase and gluco-amylase enzymes. These data showed that the enzyme concentration of 0.1% w/w (at optimal pH and temperature) was enough to convert sufficiently starch in glucose. Similar results were observed in the case of α-amylase and gluco-amylase from Valley company (data not shown).

Table 4. Sugar content of sample after ultrasound treatment.

<table>
<thead>
<tr>
<th>Time during ultrasound treatment (power =100 W)</th>
<th>Sugar content (g/100 g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td>2.37</td>
</tr>
<tr>
<td>30 min</td>
<td>3.03</td>
</tr>
</tbody>
</table>

Fermentation: Production of ethanol from sugar derived from starch and sucrose has been commercially dominated by the yeast Saccharomyces cerevisiae. The ethanol determination of fermented sample showed the optimum condition for fermentation of cassava using Saccharomyces cerevisiae at pH 5 and temperature 30°C. Up to 10% ethanol could be produce during 3 days fermentation. In general, ethanol production during fermentation of enzymatic saccharified cassava at 37°C was lower than fermentation at 30°C (Fig. 5). It was not clear if the slower fermentation rate at 37°C was because of higher temperature or incubation of this sample without agitation. An additional experiment may be necessary to investigate whether the agitation during fermentation have any acceleration effect on bioethanol production using yeast or not.

The most ethanol production (sugar conversion to bioethanol) was observed during first day of fermentation. This was true for fermentation at 30°C as well as at 37°C and different pH. Extending the fermentation time from 1 day to 3 days resulted only slight increasing in the ethanol production (Figs 5 and 6). Up to 10.5% (refractometric method) (Fig. 6) or 11% (GC method) (Fig. 7) ethanol was produced during 3 days fermentation at 30°C and pH 5.

Figure 5. Ethanol percent after fermentation measured by refractometer.

Figure 6. Ethanol percent after fermentation measured by GC.

Figure 7. pH after fermentation.
The pH of sample during 3 days fermentation remained constant independent on fermentation temperature and initial pH of sample showing no pH change during fermentation (Fig. 7).

A rest of sugar content, approximately 5 to 7.5% (from initial sugar content 23.23%), remained inside the sample after 3 days fermentation. The fermentation at pH 5 and temperature 30°C resulted the lowest sugar level remaining at 5% w/w (Fig. 8). It seems that with increasing the ethanol content in sample, the activity of yeast rapidly decreased and ethanol showed an inhibition effect on yeast. The measurement of total count confirm that at higher ethanol concentration the total count of yeast rapidly decreased from $10^{10}$ to $10^3$ and less (Table 5).

**Table 5.** Yeast count during fermentation time.

<table>
<thead>
<tr>
<th>Fermentation Condition</th>
<th>1 day</th>
<th>3 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4, 30°C</td>
<td>$3 \times 10^9$</td>
<td>0</td>
</tr>
<tr>
<td>pH 5, 30°C</td>
<td>$7 \times 10^6$</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>pH 4, 37°C</td>
<td>$1 \times 10^{10}$</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td>pH 5, 37°C</td>
<td>$4 \times 10^9$</td>
<td>$3 \times 10^3$</td>
</tr>
</tbody>
</table>

**Conclusions**

Cassava root is a good source for starch because of high starch content (24% starch in fresh sample) on fresh basis. The free sugar content in fresh cassava root is very low (0.48 g/100 g sample). Application of pectolytic/cellulolytic enzymes before saccharification increased the free sugar from 0.5% in raw material to 8% depending on enzyme used in this study. Pectolytic/cellulolytic enzyme from different companies used in this study showed different effectiveness for converting pulp to sugar. Amylolytic enzymes ($\alpha$-amylase and glucose-amylase) from company “Stern” as well as “Novozymes” showed high activity. Enzyme concentration of 0.05 to 0.1% w/w was enough to convert up to 95% starch in sugar. Amylolytic enzymes from Valley company had the highest effectivity compared to “Stern” and “Novozymes” at given enzyme concentration. Treatment of cassava root with 1 M sulphuric acid at 90°C, 2 h resulted conversion of starch to glucose but the glucose amount was less than enzymatic converted starch to glucose. The highest ethanol production was observed at 30°C fermentation temperature and pH 4 or 5. The rest of sugar content in fermented sample varied between 5 to 7.5% w/w. This sugar cannot be converted in ethanol because of inhibition effect of high ethanol concentration (higher than 9% v/v ethanol) on yeast and inactivation of yeast after three fermentation days. To convert all sugar in sample to ethanol, it is necessary to dilute the sample up to less than 20% w/w sugar concentration, or use special ethanol tolerant yeast.

**Acknowledgements**

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**References**