A Comparison of the Production of Ethanol between Simultaneous Saccharification and Fermentation and Separate Hydrolysis and Fermentation Using Unpretreated Cassava Pulp and Enzyme Cocktail

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The processes of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were employed using *Saccharomyces cerevisiae* for the production of ethanol from cassava pulp without any pretreatment. A combination of amylase, cellulase, cellobiase, and glucoamylase produced the highest levels of ethanol production in both the SHF and the SSF method. A temperature of 37 °C, a pH of 5.0, and an inoculum size of 6% were the optimum conditions for SSF. For the batch process at a pulp concentration of 20%, ethanol production levels from SHF and SSF were the highest, at 23.51 and 34.67 g L\(^{-1}\) respectively, but in the fed-batch process, the levels of ethanol production from SHF and SSF rose to 29.39 and 43.25 g L\(^{-1}\) respectively, which were 25% and 24.7% higher than those of the batch process. Thus SSF using the fed-batch provided a more efficient method for the utilization of cassava pulp.

Key words: enzyme cocktail; separate hydrolysis and fermentation; simultaneous saccharification and fermentation; ethanol; cassava pulp

Currently, ethanol is increasingly used as an alternative to fossil fuels in the transportation sector. Traditional fuel ethanol is produced from sugar cane, corn, and other kinds of starch, which are high in cost and lead to shortages and increases in the prices of food and feed. Value-added bio-products, such as fuel ethanol derived from efficient utilization of agro-industrial by-products, have attracted increased attention because of their use of abundant, renewable materials. The employment of proper bio-processing for the production of fuel ethanol is a propitious method to reduce dependence on fossil fuel and decrease environmental problems.

Cassava, the sixth most important food crop in the world due to its high level of production, is grown widely across a tropical region including Africa and South Asia. Cassava is also grown in Guangdong and Guangxi Provinces of China. Cassava pulp, a by-product of the cassava-starch industry, contains a large amount of starch (approximately 40%–60%, as measured by dry weight), as well as cellulosic fiber (approximately 10%–20% dry weight). Cassava pulp spoils rapidly and causes environmental problems, including a strong and offensive odor and contamination of the local water supply. Owing to its rich starch and cellulose content, as well as the small particle size of the lignocellulosic fibers, which helps to reduce energy- and cost-consuming procedures, such as milling and delignification, in recent years it has increasingly been employed to produce fuel ethanol through microbial reactions. Starch and fibrous components are usually hydrolyzed via acid treatment or enzymatic reactions into sugars, which can be metabolized by ethanologenic microorganisms. A low concentration of acid and a high temperature (over 120 °C) can hydrolyze cassava pulp into sugars. The employment of cellulase, pectinase, and xylanase, which help to liberate the starch granules trapped inside the cellulosic fibers, can increase the ability of α-amylase to react with the starch, which increases the efficiency of sugar production. Also, the separate hydrolysis of starch and cellulose, hemicellulose, or a combination of enzymatic hydrolysis and physical treatment in the hydrolysate can help to enhance the sugar yield. However, the prices of pectinase and xylanase are high, and common industrial ethanologenic strains, such as *Saccharomyces cerevisiae*, cannot utilize xylose (a hydrolytic product of xylanase), and hence pectinase and xylanase were not included in this study.

In the process of ethanol fuel production from cassava pulp, the methods of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) using amylase and glucoamylase are commonly employed. In the saccharification step of the SHF process, the enzymes are more likely to be inhibited by the hydrolysate than in SSF. Furthermore, the operation of SHF is more complicated than that of SSF, and the cycle is longer. Conversely, SSF is a one-step fermentation method that is simpler to perform and uses less energy. However, SHF can ensure that both the enzyme and the strain function under optimal conditions, whereas the conditions of SSF are more difficult to optimize. With the development of genetic technology...
enabling yeast strains to be constructed that express or display on their membrane surfaces enzymes that can hydrolyze pulp, \(^1^1\) a process of consolidated bio-processing (CBP) is now possible, but since the expression of this complicated enzyme system is difficult, the CBP process is often low in efficiency. Though SHF and SSF have been investigated as to the production of ethanol from cassava pulp, previous studies on the enzymatic hydrolysis of cassava pulp were usually performed at low substrate concentrations (less than 10\%) due to the insufficiency of mass and heat transfer, which is a major obstacle to industrial application, \(^1^2\)–\(^5\) and little information can be obtained as to a comparison between SHF and SSF at high solid loading. Thus a comprehensive study and comparison of the SHF and SSF processes should be made, especially at the high substrate concentrations.

In this study, SSF and SHF were compared as to ethanol production from cassava pulp based on the action of multiple enzymes and fermentation of Saccharomyces cerevisiae, and different processes of enzyme cocktail and pulp feeding were employed to improve ethanol production and fermentation efficiency at high concentrations of cassava pulp.

Materials and Methods

**Substrates.** Cassava pulp was purchased from the Wuming Starch Factory (Guangxi Province, China). Dry pulp was prepared by heating 2 kg of wet cassava pulp at 50 °C (for 48 h, followed by grinding and sieving of the pulp through a 40-mesh sieve (420 \(\mu\)m). The composition of cassava pulp was analyzed by the standard methods of the Association of Official Analytical Chemists (AOAC).\(^1^0\) The major components of the cassava pulp were 40.12\% starch, 11.40\% cellulose, 8.29\% hemicellulose, 4.43\% protein, 3.45\% fat, and 2.84\% ash (dry weight). Based on previous studies,\(^1^1\)–\(^4^1\) a concentration of cassava pulp ranging from 4\% to 20\% was chosen for both the SHF and the SSF process.

**Enzyme loading and assay determining enzyme activity.** Commercial cellulase (C) from Trichoderma reesei (Celluclast \(^1^\) 1.5L), \(\alpha\)-amylase (Amy) from Bacillus licheniformis (Termamy \(^4^2\) 120L), \(\beta\)-glucosidase (B) from Aspergillus niger (Novozyme\(^4^3\)188), and glucoamylase (AMG) from Aspergillus niger (AMG 300L) were purchased from Novozymes ( Bagsvaerd, Denmark).

Cellulase was employed at a ratio of 15 filter paper units (FPU) g\(^{-1}\) of effective cellulose (effective cellulose was the cellulose and hemicellulose contained in the cassava pulp). The FPU unit was defined and analyzed according to standard methods.\(^1^1\) The reducing sugars for cellulase activity determination were analyzed by the 3,5-dinitrosalicylic acid (DNS) method.\(^1^2\) \(\alpha\)-Amylase was used in a ratio of 500 international units (IU) g\(^{-1}\) of starch. One IU represents the quantity of enzyme required to hydrolyze 1 mg of starch per min under standard assay conditions at pH 6.0 at 70 °C with soluble starch as substrate. \(\beta\)-Glucosidase was employed in a ratio of 30 international units of \(\beta\)-glucosidase enzyme activity (CBUs) g\(^{-1}\) of effective cellulose. One CBU represents the quantity of enzyme required to produce 1 \(\mu\)mol of glucose per min under standard conditions (pH 6.0 at 70°C with soluble starch as substrate). Activity of \(\beta\)-glucosidase in an efficiency of the enzyme is determined as the amount of \(\beta\)-glucosidase required to produce 1 \(\mu\)mol of glucose per min under standard conditions at pH 5.0 at 50 °C with soluble starch as substrate. The enzyme activities of \(\beta\)-glucosidase and glucoamylase were measured by HPLC, because common sugar-determination methods, such as the DNS method, cannot distinguish disaccharide from glucose.

**Microorganism strains, media, and inoculum preparation.** The Saccharomyces cerevisiae SHY08-3 strain used in this study was obtained for ethanol production from the Jiaolong Ethanol Factory (Guangxi Province, China). The yeast was grown aerobically at 37 °C with rotary shaking at 200 rpm in yeast extract peptone dextrose medium (YPD) containing 20 g L\(^{-1}\) of glucose, 10 g L\(^{-1}\) of yeast extract, and 5 g L\(^{-1}\) of peptone. The medium was sterilized by steam autoclaving at 115 °C for 20 min, and the strain was periodically subcultured on YPD medium to maintain its activity and purity.

**Enzyme cocktail and pulp feeding.** The optimal enzyme cocktail was prepared from the culture by transferring a loop-full of active SHY08-3 cells to 10 mL of YPD medium in sterile test tubes and incubation at 37 °C at 200 rpm for 12–18 h in an incubator shaker (C24KC Refrigerated Incubator Shaker, Edison, NJ). Next, 10 mL of active cells was aseptically transferred to 100 mL of sterile YPD medium in a 250-mL Erlenmeyer flask. The flask was incubated at 37 °C at 200 rpm for 12 h in the same shaker. After cultivation, the cells were harvested by centrifugation in a 50-mL sterilized centrifuge tube (10 min at 3,000 g using a TDL-500RB centrifuge (Shanghai Anke, Shanghai, China). The cell pellets were resuspended in a sterilized 0.9% NaCl solution to obtain a cell suspension with a cell-mass concentration of approximately 30 g dry weight per liter. The time period between cell harvesting and initiation of the SHF process was no longer than 2 h. Finally, the cell suspension was employed to initiate fermentation experiments with various inoculum sizes.

**Analysis of soluble carbohydrates and ethanol.** One-mL samples taken by the experimental process were acidified with 10% sulphuric acid, centrifuged at 12,000 g for 15 min, and filtered through a membrane of 0.22 \(\mu\)m pore size. Glucose, xylose, and ethanol were identified and quantified with a Waters 2414 (Millford, LA) equipped with a refractive index detector. An Aminex HPX-87H column (300 × 7.8 mm) and a Cation H Cartridge Micro-Guard column (Bio-Rad, Hercules, CA) were employed, operating at 60 °C with 2.5 mM H\(_2\)SO\(_4\) as the mobile phase at a flow rate of 0.6 mL min\(^{-1}\).\(^1^3\)

**Optimization of enzyme cocktails for pulp hydrolysis.** Cassava pulp at a concentration of 4\% was inserted into 100-mL serum bottles. All of the serum bottles were sealed with butyl rubber stoppers and aluminium seals (as below). The pH of the suspension was pre-adjusted to 5.0, and the working volume of the system was 50 mL (as below). The suspensions were steam-sterilized at 121 °C for 30 min (as below). The filter-sterilized commercial enzymes or enzyme cocktails consisting of various combinations of enzymes (enzyme loading as outlined above) were added to the suspensions. The hydrolysis reaction was incubated at 50 °C with rotary shaking at 200 rpm for 72 h. Samples were obtained at the end of the reaction, and the total quantity of reducing sugars released and the compositions of the sugars was analyzed by the DNS method or the HPLC method.

The optimal enzyme cocktail determined by the above procedure was employed to hydrolyze the cassava pulp, which was added to the reaction at a concentration of 4\% (pH pre-adjusted to 5.0) at 50 °C with rotary shaking at 200 rpm from 24 to 144 h (time interval, 24 h) to select the optimal duration for enzymatic hydrolysis. Samples were obtained at the end of the reaction for analysis of released sugars.

**Effect of substrate concentration on the production of ethanol by the SHF procedure.** Cassava pulp at concentrations of 4\%, 6\%, 10\%, 12\%, 16\%, and 20\% was suspended in distilled water, and the pH of the suspension was pre-adjusted to 5.0. The suspensions were steam-sterilized by autoclaving at 121 °C for 30 min. The optimal enzyme cocktail, determined as above, was filter-sterilized and employed for hydrolysis. For pulp concentrations of 4\%–12\%, the reaction time was 96 h; when the pulp concentrations increased to 16\% and 20\%, the reaction time was 120 h.

Fed-batch enzymatic hydrolysis was also performed at an initial pulp concentration of 12\%, and 4\% pulp and the corresponding loading of the optimal enzyme cocktail were added to the reaction at hydrolysis time points of 24 h and 48 h respectively (after each feeding, the pH of the system was adjusted to 5.0). The end point for fed-batch hydrolysis was also 120 h.

After hydrolysis, the fermentation broth was placed in a 500-mL flask and inoculated with 5% (v:v) of a yeast culture by transferring a loop-full of active SHY08-3 cells to 10 mL of YPD medium containing 20 g L\(^{-1}\) of glucose, 10 g L\(^{-1}\) of yeast extract, and 5 g L\(^{-1}\) of peptone. The medium was sterilized by steam autoclaving at 115 °C for 20 min, and the strain was periodically subcultured on YPD medium to maintain its activity and purity.

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Effect of enzyme cocktail feeding on the process of enzymatic hydrolysis by the SHF procedure. Strategies for adding the enzyme cocktail to the reaction were investigated to improve the recovery of sugars. Feedings of the cocktail were performed as described below. (i) The initial cassava pulp concentration was 12%, and the corresponding enzymes were added to the reaction at start-up. (ii) The initial cassava pulp concentration was 12%. The corresponding enzymes were fed manually in three equally sized portions at 0 h, 24 h, and 48 h. (iii) The initial cassava pulp concentration was 8%, and the enzymes corresponding to 12% pulp were added at start-up, and those corresponding to 2% pulp were fed manually at 24 h and 48 h respectively (after every feeding of pulp, the pH was adjusted to 5.0). (iv) The initial cassava pulp concentration was 8%, corresponding loading of the enzymes was conducted at start-up, and 2% pulp and the corresponding enzymes were added at 24 h and 48 h respectively (after every feeding of pulp, the pH was adjusted to 5.0). All of the reactions were performed at 50 °C with rotary shaking at 200 rpm for 72 h. Samples were taken at the end of the reaction and analyzed as described above.

Optimization of the SSF procedure (enzyme cocktail, temperature, pH, and inoculum size). Cassava pulp at a concentration of 4% was added to 100-mL serum bottles, the pH was adjusted to 5.0, and then 3 mL of yeast (inoculum size, 6%) was inoculated, various combinations of the enzyme and the enzyme mixture being added to the suspension. The total working volume was 50 mL, as below). Ethanol fermentation was incubated at 37 °C at 200 rpm for 96 h.

At a cassava pulp concentration of 4% (pH adjusted to 5.0), the optimized enzyme cocktail described above and 3 mL of yeast were added to the reaction, and then the various reagents for ethanol fermentation were incubated at 30 °C, 35 °C, 37 °C, and 40 °C respectively at 200 rpm for 96 h.

Under the conditions of optimized enzyme cocktail and using the system at a pulp concentration of 4% and 3 mL of yeast, the system pH was adjusted to 3.0, 4.0, 5.0, 6.0, or 7.0 and the bottles were incubated at the optimal temperature described above, at 200 rpm for 96 h.

Under the conditions of optimized enzyme cocktail, temperature, and pH described above, the system at a pulp concentration of 4% was inoculated with yeast at concentrations of 4%, 6%, and 8% at 200 rpm for 96 h. These samples were obtained at the end point of fermentation and analyzed.

Effect of cassava pulp concentration on the SSF procedure. Under optimal conditions (temperature 37 °C, pH 5.0, and inoculum size 6%) for SSF, fermentation with cassava pulp at concentrations of 4%, 6%, 8%, 10%, 12%, 14%, 16%, and 20% were inoculated at 200 rpm for 120 h. Samples were obtained at the end of fermentation and analyzed.

Effect of substrate feeding on the SSF procedure. Under optimal conditions for SSF, substrate feedings were conducted to enhance the efficiency of fermentation at high concentrations of cassava pulp. Mode 1: An initial pulp concentration of 12% and enzymes corresponding to 20% of the pulp were added at start-up, and those corresponding to 4% pulp were added manually at 48 h and 96 h (after feeding, the system pH was adjusted to 5.0). Mode 2: An initial pulp concentration of 12% and the corresponding enzymes were added at start-up; 4% pulp and the corresponding enzymes were added at 48 h and 96 h respectively (after feeding, the system pH was adjusted to 5.0). For both mode 1 and mode 2, fermentation ended after 120 h and samples were taken for analysis.

Calculations and statistical method. The fermentation calculations were as follows:

Yield of released sugars (g/L/h) = \frac{\text{glucose + xylose, g L}^{-1}}{\text{pulp concentration, g L}^{-1}}

Glucose hydrolysis efficiency (%) = \frac{\text{glucose concentration, g L}^{-1}}{\text{pulp concentration} \times 51.52 \times 1.1, \text{g L}^{-1} \times 100}

Xylose hydrolysis efficiency (%) = \frac{\text{xylose concentration, g L}^{-1}}{\text{pulp concentration} \times 8.29 \times 1.1, \text{g L}^{-1} \times 100}

Fermentation efficiency (%) = \frac{\text{ethanol concentration, g L}^{-1}}{\text{pulp concentration} \times 51.52 \times 1.1, \text{g L}^{-1} \times \frac{1}{0.51} \times 100}

Because the yeast used in this study did not utilize xylose, xylose was not factored into fermentation efficiency.

Ethanol productivity (g L^{-1} h^{-1}) = \frac{\text{ethanol concentration, g/L}}{\text{hydrolysis time + fermentation time, h}}

or \frac{\text{ethanol concentration, g/L}}{\text{fermentation time, h}}

The former was for the SHF and the latter for the SSF process.

All experiments were conducted in triplicate, and data are presented as mean value ± standard deviation. Statistical analysis was carried out with Microsoft Excel by Student’s t-test, and the results were considered statistically significant at 95% confidence (p < 0.05).44

Results and Discussion

Optimization of SHF

Enzymes or enzyme cocktails consisting of various combinations of enzymes were inserted into serum bottles with 4% pulp, and this was incubated at 50 °C with rotary shaking at 200 rpm for 72 h. Neither Amy nor AMG alone, nor the combination of Amy and AMG, hydrolyzed the cassava pulp efficiently. However, when C was added to the reaction in combination with Amy and AMG, the yield of sugars reached 0.40 g g^{-1}, 1.44-fold greater than the cocktail of Amy + AMG (Table 1). For enzymatic hydrolysis of unpretreated cassava pulp, the primary obstacle was the starch granules trapped in the cellulose and hemicellulose, both of which were difficult to degrade and can act to prevent the association of starch and enzyme. Thus C played a critical role in hydrolysis. Cellobiose, an inhibitor of cellulase, can be hydrolyzed by B to glucose. Hence, after the mixture of C + B + Amy + AMG, the yield reached 0.46 g g^{-1}, an increase of 15% compared with that of C + Amy + AMG. These results indicate as positive synergism between C + B and Amy + AMG.

No significant increases were observed in the levels of released sugars after 72 h of hydrolysis (Table 2), and hence 72 h was chosen as the most efficient enzymatic hydrolysis time. This result was probably due to inhibition by end products and by-products of the hydrolysis reaction40 and/or pH changes,13 which might have resulted in reduced activity of the enzymes. The removal of end products via filtration induced a 20% increase in production in a study by Chotineeranat et al.9
Most previous studies investigating the hydrolysis of cassava pulp examined the hydrothermal reaction using H₂SO₄ or HCl and high temperature of over 100 °C, and the yields were usually high. Kosugi et al. reported that glucose hydrolysis efficiency was approximately 90% of the theoretical value (with a starch content of 60.6% instead of the 40.12% found in the present study) under hydrothermal conditions (140 °C for 1 h). This is approximately 15% higher than the highest glucose hydrolysis efficiency obtained in the present study, but no fermentation-inhibiting by-products, such as furfural or hydroxymethylfurural, were detected in the hydrolysate from the enzymatic hydrolysis process in the present study. These were expected to be produced during the acid hydrolysis process and might have exerted a significantly negative effect (furfural at low concentrations of 1–12 mm and hydroxymethylfurural over 10 mm) on the subsequent fermentation reaction. In the enzymatic hydrolysis process, the addition of xylanase, pectinase, and laccase, which facilitate the degradation of hemicellulose and the lignin bundle, enhanced enzymatic efficiency. The sugar yields reported by Nair et al. (C + xylanase + hemicellulase) and Rattanachomrsri et al. (C + B + AMG + Amy + Pectinase) were 0.57 g g⁻¹ dry pulp, approximately 20% higher than our result due to an approximately 50% higher starch content of 60.6%, instead of the 40.12% found in the present study and pre-treatment.

Effect of cassava pulp concentration on ethanol production by the SHF procedure

For industrial application, the substrate concentration must enhanced to reduce operating costs, but highly concentrated cassava pulp represents a challenge because it is resistant to heat and mass transfer and tends to decrease the efficiency of hydrolysis. Thus it is important to find a suitable substrate concentration. Cassava pulp at concentrations of 4%, 6%, 10%, 12%, 16%, and 20% was produced.

The concentrations of glucose and xylose rose with increases in the pulp concentration. The levels of glucose and xylose were 52.70 g L⁻¹ and 6.01 g L⁻¹ respectively at a pulp concentration of 20% (Table 3). However, hydrolysis efficiency and Y∝/S decreased from 74.40% and 0.46 g g⁻¹ to 46.46% and 0.29 g g⁻¹ respectively with increases in the pulp concentration. Also, increased pulp concentrations caused the pH value after hydrolysis to increase from 4.8 in 20% to 4.0 in 4%, which caused the pH to deviate from the optimum range (approximately 5.0) for the enzymes, resulting in low hydrolysis efficiency and Y∝/S. Significant increases in glucose hydrolysis efficiency and Y∝/S were observed after adjustment of the pH to approximately 5.0 and the addition of pulp and enzymes to the reaction by the fed-batch process, which confirms the hypothesis stated above. The glucose production generated by fed-batch hydrolysis was as high as 66.86 g L⁻¹. In addition, when a final pulp concentration of 20% was employed, an increase of 26.9% was observed as compared with batch hydrolysis.

### Table 1. Yields of Sugars Released from Cassava Pulp Hydrolyzed with Various Combinations of Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>C (FPUG⁻¹)</th>
<th>B (CBGU⁻¹)</th>
<th>AMG (IU g⁻¹)</th>
<th>Amy (IU g⁻¹)</th>
<th>Y∝/S (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amy</td>
<td>—**</td>
<td>—</td>
<td>—</td>
<td>500</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>AMG</td>
<td>—</td>
<td>—</td>
<td>500</td>
<td>—</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Amy + AMG</td>
<td>15</td>
<td>30</td>
<td>—</td>
<td>500</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>C + B</td>
<td>15</td>
<td>30</td>
<td>—</td>
<td>500</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>C + Amy</td>
<td>15</td>
<td>30</td>
<td>—</td>
<td>500</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>C + AMG + Amy</td>
<td>15</td>
<td>30</td>
<td>—</td>
<td>500</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>C + B + Amy + AMG***</td>
<td>15</td>
<td>30</td>
<td>—</td>
<td>500</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>n.d.***</td>
</tr>
</tbody>
</table>

**Yield of released sugars.**

**Enzyme cocktail:**
- Amy: amylase
- AMG: xylanase
- C: cellulase
- **:** not added.

### Table 2. Time Course of Hydrolysis of Cassava Pulp with Enzyme Cocktail

<table>
<thead>
<tr>
<th>Hydrolysis time</th>
<th>Glucose (g L⁻¹)</th>
<th>Glucose productivity (g glucose L⁻¹ h⁻¹)</th>
<th>Xylose (g L⁻¹)</th>
<th>Y∝/S (g g⁻¹)</th>
<th>GHE (%)</th>
<th>XHE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>26.11 ± 1.44</td>
<td>1.09 ± 0.03</td>
<td>0.86 ± 0.03</td>
<td>0.34 ± 0.16</td>
<td>57.68 ± 3.19</td>
<td>11.14 ± 0.74</td>
</tr>
<tr>
<td>48h</td>
<td>31.37 ± 1.64</td>
<td>0.65 ± 0.02</td>
<td>1.73 ± 0.02</td>
<td>0.41 ± 0.19</td>
<td>69.22 ± 3.63</td>
<td>22.43 ± 2.74</td>
</tr>
<tr>
<td>72h</td>
<td>33.20 ± 0.57</td>
<td>0.46 ± 0.01</td>
<td>2.52 ± 0.01</td>
<td>0.45 ± 0.06</td>
<td>73.20 ± 1.25</td>
<td>32.65 ± 0.55</td>
</tr>
<tr>
<td>96h</td>
<td>33.34 ± 0.48</td>
<td>0.35 ± 0.003</td>
<td>2.74 ± 0.003</td>
<td>0.45 ± 0.05</td>
<td>73.58 ± 1.06</td>
<td>35.58 ± 0.40</td>
</tr>
<tr>
<td>120h</td>
<td>33.54 ± 0.31</td>
<td>0.28 ± 0.001</td>
<td>2.92 ± 0.001</td>
<td>0.45 ± 0.04</td>
<td>74.01 ± 0.69</td>
<td>37.87 ± 0.02</td>
</tr>
<tr>
<td>144h</td>
<td>33.70 ± 0.07</td>
<td>0.23 ± 0.002</td>
<td>2.95 ± 0.002</td>
<td>0.46 ± 0.01</td>
<td>74.32 ± 0.16</td>
<td>38.32 ± 0.015</td>
</tr>
</tbody>
</table>

**Hydrolysis was conducted at 50 °C at an initial pH of 5.0 with rotational shaking at 200 rpm for 72 h with 4% cassava pulp.**

**Yield of released sugars.**

**Y∝/S:** yield of released sugars.

**GHE:** glucose hydrolysis efficiency.

**XHE:** xylose hydrolysis efficiency.
The level of ethanol production increased with increasing pulp concentrations. When the pulp concentration reached 20%, the ethanol concentration rose to 23.51 g L\(^{-1}\). The ethanol concentration reached 29.39 g L\(^{-1}\) at a 20% pulp concentration for the fed-batch process, which represents an increase of 25% as compared with the batch process. The highest fermentation efficiency, of 68.19% (Table 4), was achieved at a pulp concentration of 4%. In view of the fermentation efficiency and the hydrolysis time, a pulp concentration of 12% was selected as the optimum substrate concentration for a one-step hydrolysis procedure for the slow ethanol concentration increase rate and the significant decrease in fermentation efficiency when the pulp concentration was over 12%. When the substrate concentration was over 12%, the fed-batch process was preferable in order to increase the levels of sugar recovery and ethanol.

Effect of enzyme cocktail feeding on the process of enzymatic hydrolysis by the SHF procedure

Four strategies for enzyme cocktail and substrate feeding were tested to improve recovery. Both the \(\text{Y}_{\text{R/S}}\) values and the glucose production levels for modes B, C, and D were higher than those for mode A (Table 5), which indicates that enzyme cocktail feedings can increase glucose production and \(\text{Y}_{\text{R/S}}\). The glucose production level and \(\text{Y}_{\text{R/S}}\) for mode D reached 50.01 g L\(^{-1}\) and 0.47 g g\(^{-1}\) respectively, which represents increases of 13.9% and 17.5% respectively as compared to those for mode A. In the process of enzymatic hydrolysis, inhibition by end products and loss of enzyme activity were increasingly important, as enzyme and substrate feedings suppressed inhibition and reduced enzyme loading at constant substrate concentrations. Glucose production and \(\text{Y}_{\text{R/S}}\) for mode B were slightly higher than those for mode C. Compared with mode B, the lower initial pulp concentration in mode D, which improved the mass and heat transfer of the enzymatic hydrolysis, resulted in higher levels of released sugars. In the time-consuming process of enzymatic hydrolysis, enzyme feeding resulted in improved recovery of sugars. Ethanol fermentation demonstrated that improved recovery of sugars resulted in higher ethanol production.

Optimization of SSF (enzyme cocktail, temperature, \(\text{pH}\), and inoculum size)

Optimization was achieved at a cassava pulp concentration of 4% at an inoculum size of 6% and incubation at 37°C at 200 rpm for 96 h. Both the level of ethanol was preferable in order to increase the levels of sugar concentration was over 12%, the fed-batch process was.
en.png

Table 5. Enzymatic Hydrolysis of Cassava Pulp by Substrate and Enzyme Feeding

<table>
<thead>
<tr>
<th>Mode</th>
<th>Glucose (g L⁻¹)</th>
<th>Glucose productivity (g glucose L⁻¹ h⁻¹)</th>
<th>Xylose (g L⁻¹)</th>
<th>Y_{R,S} (g g⁻¹)</th>
<th>GHE** (%)</th>
<th>XHE*** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>43.90 ± 1.06</td>
<td>0.61 ± 0.01</td>
<td>4.35 ± 0.28</td>
<td>0.40 ± 0.01</td>
<td>64.61 ± 1.56</td>
<td>37.61 ± 1.22</td>
</tr>
<tr>
<td>B</td>
<td>48.12 ± 1.09</td>
<td>0.67 ± 0.01</td>
<td>5.13 ± 0.11</td>
<td>0.45 ± 0.01</td>
<td>70.88 ± 1.60</td>
<td>44.26 ± 0.49</td>
</tr>
<tr>
<td>C</td>
<td>47.66 ± 2.18</td>
<td>0.66 ± 0.02</td>
<td>4.13 ± 0.07</td>
<td>0.44 ± 0.02</td>
<td>70.46 ± 3.20</td>
<td>35.71 ± 0.31</td>
</tr>
<tr>
<td>D</td>
<td>50.01 ± 1.44</td>
<td>0.69 ± 0.02</td>
<td>5.88 ± 0.17</td>
<td>0.47 ± 0.01</td>
<td>73.52 ± 2.12</td>
<td>50.78 ± 0.74</td>
</tr>
</tbody>
</table>

Hydrolysis was conducted at 50 °C at an initial pH of 5.0 with rotational shaking at 200 rpm for 72 h. Mode A. Initial cassava pulp concentration of 12% and corresponding loading of the enzymes. B. Initial cassava pulp at a concentration of 12% was added at start-up, and the enzymes were fed manually in three equally sized portions at 0 h and 24 h, and 48 h. C. Initial cassava pulp concentration of 8% and the enzymes corresponding to 12% pulp were added at start-up. Pulp at 4% was fed manually in two equally sized portions at 24 h and 48 h. D. Initial cassava pulp concentration of 8%. The corresponding loading of the enzymes were added at start-up, and 2% pulp and the corresponding enzymes were added at 24 h and 48 h respectively.

*Y_{R,S}. yield of released sugars.
**GHE, glucose hydrolysis efficiency.
***XHE. xylose hydrolysis efficiency.

production and the efficiency of fermentation were highest, at 7.71 g L⁻¹ and 70.76% respectively, when all four of the enzymes were added (Fig. 1A). The ethanol production of 7.31 g L⁻¹ from C + AMG + Amy was similar to that of C + B + Amy + AMG, which caused increases of 38% and 40% respectively as compared to those of the single addition of C and the combination Amy + AMG. The combined actions of cellulase and amylase produced larger quantities of ethanol, owing to efficient hydrolysis of the non-starch fibrous structure. The addition of B to the enzyme cocktail did not result in a significant increase in ethanol, perhaps due to the presence of β-glucosidase activity in the commercial cellulase (approximately 26.43 CBU mL⁻¹). This finding differs from the report of Rattanachomsri.10

In the SSF method, the goal was to identify the optimal enzyme combination and optimal fermentation of the yeast. The yeast SHY08-3 strain did not grow well at temperatures over 40 °C, but the optimal temperature for the enzyme cocktail was 50 °C. The highest ethanol production, of 7.71 g L⁻¹, and a fermentation efficiency of 70.76% were achieved at 37 °C (Fig. 1B). Neither a decrease nor an increase in the temperature induced a significant decrease in ethanol production or fermentation efficiency. These results indicate that the balance between fermentation temperature and enzyme hydrolysis was the key factor for ethanol production in this SSF study. In future studies, the use of thermo-stable yeast strains by the SSF method might improve ethanol yields.

The optimal pH and inoculum size by the SSF method were 5.0 and 6% respectively (Fig. 1C, D). Under these optimal conditions, almost all the glucose released from the cassava pulp was fermented into ethanol (data not shown).

Effect of cassava pulp concentration on the SSF procedure

Cassava pulp at concentrations of 4%, 6%, 8%, 10%, 12%, 14%, 16%, and 20% was incubated at 200 rpm for 120 h. The level of ethanol production increased with
increasing pulp concentrations. At a pulp concentration of 20\%, the ethanol concentration was 34.67 g L$^{-1}$, nearly 4.5 times of that of the pulp concentration of 4\%.

A high pulp concentration can lead to a high ethanol concentration, resulting in improved efficiency of downstream processing, but the efficiency of ethanol fermentation decreased with increases in the pulp concentration, ranging from the highest efficiency of 70.76\% at 4\% pulp to the lowest of 63.60\% at 20\% pulp (Table 4). When the pulp concentration increased to over 16\%, the operation was difficult and a significant decrease in fermentation efficiency was observed, due to the difficulty of heat and mass transfer at high solid loading.\(^{20,21}\) Thus a pulp concentration of 16\% was most suitable for ethanol production by the batch SSF method based on ethanol production, fermentation efficiency, and the simplicity of the operation.

**Effect of substrate feeding on the SSF procedure**

Two modes of substrate and enzyme cocktail feedings were performed. Compared with batch fermentation at a pulp concentration of 20\%, fed-batch fermentation significantly improved the efficiency of fermentation and the production of ethanol. The ethanol production level and fermentation efficiency for mode 1 were 40.12 g L$^{-1}$ and 71.39\% respectively, which represent increases of 15.7\% and 12.2\% respectively as compared with those batch fermentation (ethanol production of 34.67 g L$^{-1}$ and fermentation efficiency of 63.60\%). The highest ethanol production level, of 43.25 g L$^{-1}$, and a fermentation efficiency of 76.96\% were achieved in mode 2, representing increases of 1.25- and 1.21-fold of those of batch fermentation, respectively. The fed-batch process reduced the solid concentration in the initial broth and enhanced the efficiencies of mass and heat transfer, causing the hydrolysis and fermentation reactions to be faster and more efficient. Ethanol production and fermentation efficiency for mode 2, with fewer enzymes at start-up, both increased by 7.8\% as compared with those for mode 1. This indicates that the enzyme feedings were beneficial for ethanol production in the time-consuming process of hydrolysis and fermentation, consistently with the conclusion drawn in the SHF in this study. Enzyme feeding might be helpful for the stability of the enzymes, which might decrease with ethanol production, pH change, by-products as well as temperature.\(^{22}\) The synergistic effect of these factors on the enzymes at high solid loading might be more severe than those reported.

**Comparison of SHF and SSF**

The SSF process exhibited notable advantages over the SHF method with increasing cassava pulp concentrations. The ethanol concentration, fermentation efficiency, and ethanol productivity of a 20\% cassava pulp substrate by the SSF method increased by 47\%, 47\%, and 107\% respectively as compared with those by the SHF method. Despite a substantially lower starch content, these results were also higher than those reported by Kosugi \cite{et} Table 4 summarizes the results of a comparison of the SSF and SHF methods.

Our results indicate that SSF is a preferable process configuration for the fermentation of ethanol from cassava pulp at high solid concentrations, in accordance with the results reported by Öhgren \cite{et} regarding corn stover. However, fermentation of 20\% cassava pulp by the batch SSF method resulted in only 0.29 g L$^{-1}$ h$^{-1}$ ethanol productivity and 63.60\% fermentation efficiency, much lower values than those reported by Rattanachomski \etal.\(^{10}\) Notably, cassava pulp with a much higher starch content and a lower cassava pulp concentration was used in this prior research (4\% instead of the 20\% used in the present study).

Fed-batch SSF with cassava pulp and enzyme feeding resulted in an economically recoverable ethanol concentration (greater than 40 g L$^{-1}$) of 43.25 g L$^{-1}$, which represents increases of 25\% and 47\% respectively compared with those for the batch SSF and the fed-batch SHF methods. Ethanol productivity further increased, from 0.29 g L$^{-1}$ h$^{-1}$ for batch SSF to 0.36 g L$^{-1}$ h$^{-1}$ using cassava pulp and enzyme feeding. We found the fed-batch operating method used in this study to be a satisfactory method of ethanol production from cassava pulp at high solid concentrations. At the ethanol concentration achieved in this study, a relatively favorable energy balance of ethanol recovery can be obtained.\(^{23}\)

Previous studies on the enzymatic hydrolysis of cassava pulp for ethanol production were usually performed at low substrate concentrations (less than 10\%), which is beneficial for the improvement of hydrolysis and the enhancement of fermentation efficiency, but increases the difficulties for potential industrial application. Rattanachomski \etal.\(^{10}\) reported a highest ethanol production, of 14.3 g L$^{-1}$, at 4\% pulp. Similarly, Akaracharanya \etal.\(^{15}\) reported a highest ethanol concentration, of 11.9 g L$^{-1}$, at 3\% pulp. Fermentation efficiency decreased significantly with increasing pulp concentrations. A study by Kosugi \etal.\(^{11}\) indicated that ethanol production by fermentation with surface-engineered yeast displaying glucoamylase at 30\% pre-treated pulp reached an ethanol production level as high as 42.1 g L$^{-1}$. Considering the elevated starch content (60.10\% as opposed to 40.12\% in the present study) and the increased cassava pulp loading of the above study (30\% as opposed to 20\% in the present study), the fed-batch strategy used in this study appears to be efficient for the production of ethanol from cassava pulp.

These promising results indicate that the bioconversion of cassava pulp to ethanol is efficient, even without pre-treatment and detoxification, and that SSF has advantages over SHF in ethanol production from cassava pulp. Due to insufficient heat and mass transfer, which causes inefficiency in both process at high concentrations of cassava pulp,\(^{10,21}\) the ethanol concentration from cassava pulp fermentation was low in previous studies. The present study solved this problem by reasonable feeding of the substrate and enzyme cocktail, and the ethanol concentration was enhanced significantly. The fed-batch SSF method with substrate and enzyme feeding promises industrial applications in ethanol production at high levels of cassava pulp loading.

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References