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ORIGINAL ARTICLE

Evaluation of the waste from cassava starch production as a substrate for ethanol fermentation by *Saccharomyces cerevisiae*

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Abstract Cassava starch production waste (cassava pulp) has been proposed as a high potential ethanolic fermentation substrate due to its high residual starch level and the small particle size of the lignocellulosic fibers. Saccharification of the residual starch from a 3% (w/v) dry weight basis (DS) of cassava pulp by α-amylase (100°C, 10 min) and glucoamylase (60°C, 2 h) resulted in a glucose yield of 22.6 g/l [67.8% (w/w) DS of cassava pulp] and in lignocellulosic fibers at 0.5 g/g DS cassava pulp. Pretreatment of the lignocellulosic fiber with dilute sulfuric acid and calcium hydroxide at 121°C, 15 lb/in^2 for 30 min increased and decreased, respectively, its susceptibility to cellulase hydrolysis. Under the optimal conditions found, pretreatment of 6% (w/v) DS lignocellulosic fiber by 2% (w/v) H_2SO_4 for 30 min, followed by saccharification by cellulase (40°C, 9 h), yielded a glucose level of 26.6 g/l [79.8% (w/w) DS of the cassava pulp]. The starch and lignocellulosic fiber hydrolysates obtained from 30 g cassava pulp and 60 g H_2SO_4 pretreated lignocellulosic fiber were fermented by *Saccharomyces cerevisiae*, without the need for $(NH_4)_2SO_4$ supplementation, to yield ethanol levels of 9.9 and 11.9 g/l, respectively, after 48 h.

Keywords Cassava pulp · Ethanol · Starch · Lignocellulose

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Introduction

Cassava (*Manihot esculenta* Crantz) is one of the major commercial crops in Thailand, with an annual production of approximately 23 million tons of cassava root. One-half of this cassava root crop is exported as cassava chips and pellets that are used principally for ethanol fuel production. The remainder is supplied as a raw material for cassava starch production, which alone generates approximately 1.7 million tons of solid cassava pulp waste after the starch extraction (Office of the National Economic and Social Development Board 2007). However, the starch extraction from cassava roots in Thailand is relatively inefficient, resulting in waste cassava pulp typically still being comprised of some 60–70% (w/w) dry weight basis (DS) starch and 15–30% (w/w) DS lignocellulosic fiber (Balagopalan et al. 1994; Sriroth et al. 2000). Due to the high starch and moisture content [75–80% (w/w) DS], the cassava pulp spoils rapidly causing environmental problems, including a strong and offensive putrefaction odor and local water contamination. If the starch in cassava pulp could be hydrolyzed economically to fermentable sugars, and prin-

cipally glucose, it would not only help solve the environmental problem of cassava pulp disposal but also provide added value for cassava crops, which are typically grown by small-scale farmers in poor or developing regions. Moreover, the lignocellulosic fiber obtained after the starch is removed from the cassava pulp is potentially an ideal substrate for ethanol production. Since it has a 20–40 mesh particle size, cutting and milling processes are not needed, saving on the requirement for these energy- and cost-consuming steps that serve as a major constraint for lignocellulosic ethanol production from other substrates. In contrast to the above-suggested potential use, the actual current use of cassava pulp, if not wasted, is either as a low-grade animal feed or for fertilizer. The improved utilization of cassava pulp is, therefore, an important and necessary step towards efficient cassava root usage.

In this work, both the starch and the lignocellulosic fibers in waste cassava pulp were hydrolyzed enzymatically to glucose and then fermented to ethanol by *Saccharomyces cerevisiae*. The susceptibility of the lignocellulosic fiber to cellulase after pretreatment with either dilute sulfuric acid or calcium hydroxide was compared. Potential inhibitors of the ethanolic fermentation by *S. cerevisiae* that were liberated from the pretreatment step were analyzed. From the results presented here, cassava pulp is proposed as a potentially promising, sustainably renewable, plentiful and low cost substrate for ethanol production.

Materials and methods

Cassava pulp

Cassava pulp was collected from Sanguan Wongse Industries, Nakhonratchashima Province, Thailand, in a frozen form and was thawed to room temperature just prior to use. The major components, on a dry weight basis (DS) were 67.8% (w/w) starch and 11.2% (w/w) lignocellulosic fiber but, as obtained, the moisture content was 80% (w/w) wet weight.

Microorganism

Saccharomyces cerevisiae TISTR 5596 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). A single colony of *S. cerevisiae* was grown in yeast peptone dextrose medium (YPD; yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l, pH 4.5) at 30°C with shaking (150 rpm) for 48 h, and then transferred [1% (v/v) inoculum] to cassava pulp-starch hydrolysate supplemented with 0.2% (w/v) $(NH_4)_2SO_4$ and incubated at the same conditions as above. This was grown to late log phase and was then used as the *S. cerevisiae* inoculum.

Saccharification of starch in cassava pulp

Cassava pulp was suspended in water at 3% (w/v) DS and saccharified by α-amylase (8.4 units/μl; Genecor International, Rochester, NY), as per the supplier's instructions, using 58.8 U/g DS at 100°C for 10 min. Under these conditions, the cassava pulp slurry did not turn blue after the addition of iodine solution, indicating the hydrolysis of most to all of the starch. The cassava pulp slurry was then further saccharified by glucoamylase (0.4 units/μl; Genecor), as per the supplier's instructions, using 2 U/g DS at 60°C for 2 h. The hydrolysate so obtained was separated from the residual lignocellulosic fiber by filtration through a stainless filter (0.88×10^3 μm pore size) and centrifuged (11,500 *g* for 20 min) at 4°C. The supernatant, referred to as the starch hydrolysate, was harvested and analyzed for reducing sugar and glucose content (see Analytical procedures), whilst the pellet, the lignocellulosic fiber, was treated as described in the next section.

Saccharification of lignocellulosic fiber in cassava pulp

The lignocellulosic fiber, separated from the starch hydrolysate, was pretreated by suspending at 3% (w/v) DS in the indicated concentration of either sulfuric acid [0–2.5% (w/v)] or calcium hydroxide [0–1.5% (w/v)] solution and autoclaved at 121°C, 15 lb/in^2 for 30 min. The pretreated lignocellulosic fiber suspension was then adjusted to pH 6 and saccharified by cellulase GC 220 (6.2 units/μl; Genecor), as per the supplier's instructions, using 62 U/g DS at 40°C with shaking (150 rpm) for 72 h. After filtration and centrifugation, as detailed above, to separate the hydrolysate from the remaining lignocellulosic fibers, the hydrolysate was analyzed for reducing sugar and glucose content. An optimal condition for this pretreatment step was determined by individually varying the concentration of sulfuric acid or calcium hydroxide solution, the percentage of lignocellulosic fiber loaded [3, 6 and 8% (w/v)] and the autoclaving period (15, 30 and 45 min). The optimal saccharification period of the pretreated lignocellulosic fiber with cellulase GC 220 at 62 U/g DS was also determined (0–72 h).

Detoxification of lignocellulosic fiber-acid hydrolysate

The lignocellulosic fiber-acid hydrolysate was detoxified by the method described by Gupta et al. (2009). Calcium hydroxide was added to the acid hydrolysate at room temperature with constant stirring until the pH reached 10 and then held with stirring for 30 min. Then, the detoxified hydrolysate was neutralized with concentrated H_2SO_4 and centrifuged at 4°C, 10,000 *g* for 15 min to remove the precipitate formed during the neutralization stage.

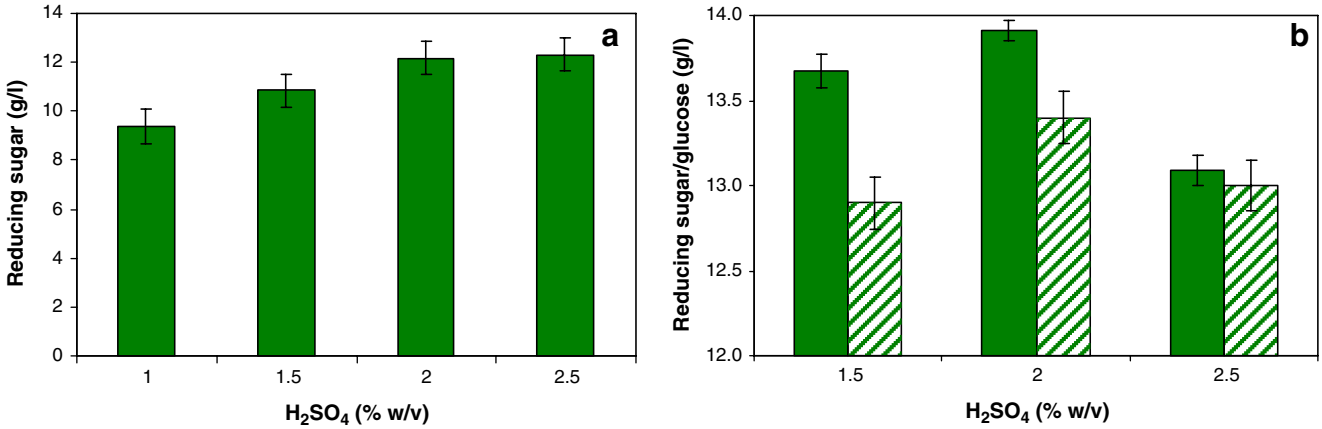

Fig. 1 Effect of H_2SO_4 concentration in the acid pretreatment on **a** the subsequent level of the reducing sugar liberated in pretreatment hydrolysate, and **b** the degree of susceptibility of the pretreated lignocellulosic fiber to cellulase degradation (hydrolysis). Lignocellulosic fibers [3% (w/v) DS] were pretreated by various concentrations of H_2SO_4 at 121°C, 15 lb/in^2 for 30 min, then saccharified by cellulase GC 220 (62 U/g) at 40°C, pH 6 for 72 h. *Solid bars* Reducing sugar level, *hatched bars* glucose level. The data are displayed as the mean±1 SD, and are derived from triplicate experiments

Ethanol fermentation

A late log phase *S. cerevisiae* culture (1.96×10^7 cell/ml) was inoculated at 10% (v/v) into the starch or lignocellulosic fiber hydrolysate and incubated at 30°C, pH 4.5 without shaking for 72 h. Different levels of ammonium sulfate [$(NH_4)_2SO_4$; 0–0.6% (w/v)] supplementation, and fermentation periods (0–72 h), were evaluated for optimization.

Analytical procedures

The cellulose, hemicellulose and lignin contents were determined by the method described by the Technical Association of Pulp and Paper Industry method (TAPPI 1988). Reducing sugars were quantified using the Somogyi-Nelson method (Somogyi 1952). Sugars and pretreatment byproducts (furfural, hydroxymethylfurfural, 4-hydroxybenzaldehyde, syringaldehyde and vanillin) were analyzed by HPLC (Agilent 1100 Series equipped with quaternary pump, on-line degasser, autoinjector, column thermostat, refractive index detector and a ChemStation softwares, Agilent Technologies, Wilmington, DE). Sugars were identified and quantified by Aminex column HPX-87P (300×7.8 mm) with a Carbo-P micro-guard cartridge (Bio-Rad, Richmond, CA). The Column was maintained at 80°C and 20 µl of each sample was injected at a time and eluted with Milli-Q filtered water at a flow rate of 0.6 ml/min The pretreatment byproducts were identified and quantified by Aminex column HPX-87 H (300 × 7.8 mm) with a Cation H micro-guard cartridge (Bio-Rad). The column was maintained at 5°C and 50 µl of each sample was injected at a time and eluted by 0.01 M $(NH_4)_2SO_4$ at a flow rate of 0.6 ml/ min.

Ethanol was quantified by GC (Hewlett-Packard, HP 5890 Series) with an Porapak QS (Cabowax 20 M) column

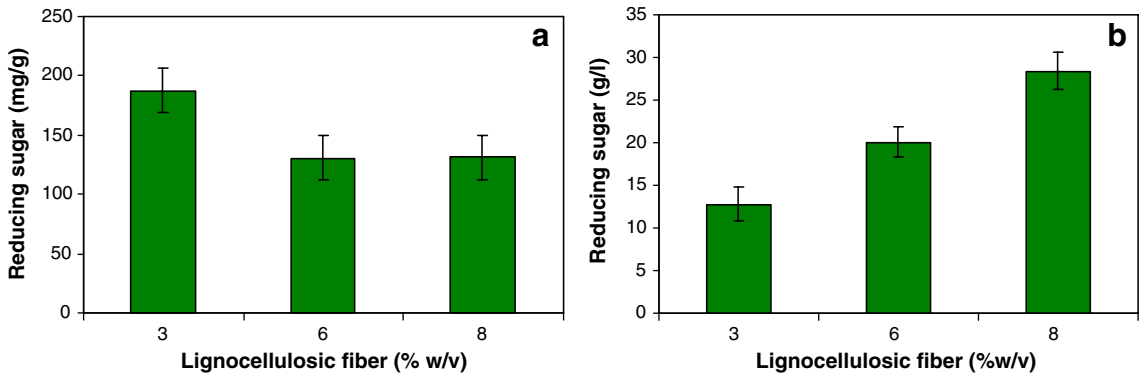

Fig. 2 Effect of the lignocellulosic fiber loading levels in the 2% (w/v) H_2SO_4 pretreatment on the subsequent level of the reducing sugar liberated, shown as **a** the reducing sugar liberation efficiency and **b** the reducing sugar concentration (yield) obtained. The data are displayed as the mean±1 SD, and are derived from triplicate experiments

Table 1 Sugars and byproducts from H_2SO_4 and $Ca(OH)_2$ pretreatment of lignocellulosic fibers. Lignocellulosic fiber [6% (w/v)] was pretreated by 2% (w/v) H_2SO_4 or 0.1% (w/v) $Ca(OH)_2$ at 121°C, 15 lb/in^2 for 30 min. The results are displayed as the mean±1 SD, and are derived from triplicate determinations. *HMF* Hydroxymethylfurfural

	Pretreatment byproducts(g/1)						
Pre-treatment	Glucose	Xylose	Purpural	HMF	Syringaldehyde	4-Hydroxybenzaldehyde	Vanillin
H_2SO_4	8.4±0.01	8.2±0.06	-	-	-	-	-
$Ca(OH)_2$	0.16±0.01	0.08±0.01	-	-	-	-	0.02±0.0003

-Not detected

(2 m×0.32 m) at an oven temperature of 175°C and a flame ionization detector (FID) at 150°C. Helium, with a flow rate of 35 ml/min, was used as the carrier gas.

Results and discussion

Saccharification of starch in cassava pulp

Analysis of the starch hydrolysates, obtained from a 3% (w/v) DS suspension of cassava pulp saccharified by α-amylase and glucoamylase as detailed in Materials and methods, revealed that the obtained glucose levels increased with increasing glucoamylase levels from 0.4 U/g (11.8 g glucose/l) to a maximal glucose yield of 22.6 g/l in the presence of glucoamylase 2 U/g. Indeed, the 23.5 g/l reducing sugar and 22.6 g/l glucose seen in the hydrolysate under these conditions represents some 68–70% (w/w) of the total mass of the cassava, and is close to the 68% (w/w) starch composition, suggesting a high degree of saccharification. This starch hydrolysate, once separated from the lignocellulosic fiber by filtration and centrifugation, was fermented to ethanol by *S. cerevisiae*, while the lignocellulosic fiber was further pretreated (see next section) and then saccharified by cellulase to glucose.

Saccharification of lignocellulosic fiber in cassava pulp

Saccharification of cassava pulp (3 g DS) by α-amylase and glucoamylase generated 1.5 g dry weight of lignocellulosic fiber, which was found to consist of 40% (w/w) cellulose, 18% (w/w) hemicellulose and 9% (w/w) lignin. Pretreatment of the lignocellulosic fiber mass by suspending [3% (w/v) DS] in various concentrations of H_2SO_4 indicated that the lignocellulosic fiber was most susceptible to cellulase GC 220 saccharification after treatment with 2% (w/v) H_2SO_4 at 121°C, 15 lb/in^2 for 30 min (Fig. 1). Increasing the amount of lignocellulosic fiber loaded from 3% (w/v) to 6% (w/v), reduced the amount of sugar liberated per gram of substrate some 1.5-fold but this then remained stable at ~125 mg / g as the fiber loading level was further increased up to 8% (w/v) (Fig. 2a). In contrast, the reducing sugar concentration in the hydrolysate increased 1.6- and a further 1.4-fold as the amount of lignocellulosic fiber loaded was increased from 3% (w/v) to 6 and 8% (w/v), respectively (Fig. 2b).

A lignocellulosic fiber loading of 6% (w/v) DS was thus selected based upon the homogeneity of the substrate during pH adjustment and the degree of cellulase saccharification. The reducing sugar level obtained from pretreatment of the 6% (w/v) DS lignocellulosic fiber, suspended in 2% (w/v) H_2SO_4 at 121°C, 15 lb/in^2, reached a maximal level of 22 g /l after 30 min of treatment (being only ~21.6 and ~21.7 g/l at 15 and 45 min autoclaving, respectively) where, at this time, the fermentation inhibitory byproducts of furfural, hydroxymethylfurfural, 4-hydroxylbenzaldehyde, syringaldehyde and vanillin, all remained below the detection thresholds of these assays (Table 1). The glucose yield obtained after saccharification of the H_2SO_4 pretreated lignocellulosic fiber by cellulase GC 220 increased with increasing hydrolysis time from 15 g/l at 0 h to a maximum glucose yield of 26.6 g/l at 9 h, and remained at this level at

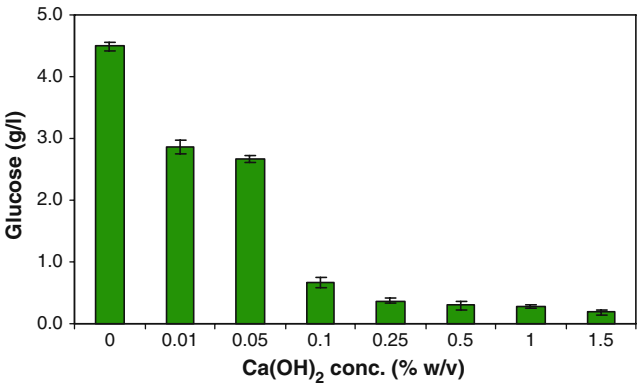

Fig. 3 Effect of $Ca(OH)_2$ concentration on the subsequent susceptibility of the lignocellulosic fibers to cellulase degradation (hydrolysis). Lignocellulosic fibers (6% (w/v)) were pretreated with the indicated concentration of $Ca(OH)_2$ at 121°C, 15 lb/in^2 for 30 min, and then hydrolyzed by cellulase GC 220 (62 U/g) at 40°C, pH 6 for 72 h. The data are displayed as the mean±1 SD, and are derived from triplicate experiments

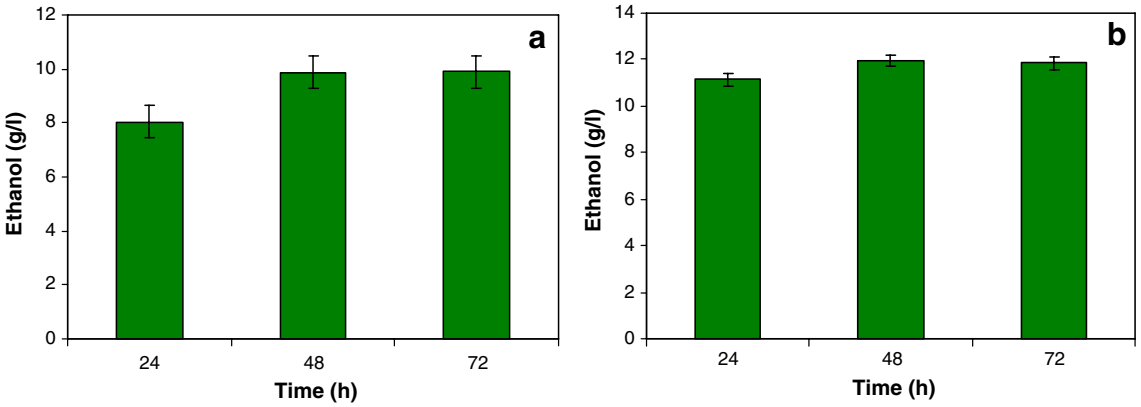

Fig. 4 Effect of the fermentation period on the level of ethanol production from **a** the starch hydrolysate, and **b** the H_2SO_4 / cellulase treated lignocellulosic fiber [6% (w/v) loaded] hydrolysate, as substrates. Hydrolysates were fermented without $(NH_4)_2SO_4$ supplementation to ethanol by *S. cerevisiae* at 30°C, pH 4.5 without shaking. The data are displayed as the mean±1 SD, and are derived from triplicate experiments

all longer time points up to 72 h. This 26.6 g/l glucose containing hydrolysate, equivalent to 79.8% (w/w) of the dry cassava pulp, was then fermented to ethanol (see Ethanol fermentation).

This glucose yield reported here is considerably higher than that reported previously by Srinorakutara et al. (2006), who reported a yield of 6.2% (w/v) of reducing sugar after treatment of cassava pulp [65.37% (w/w) DS starch content] at an initial substrate loading of 11% (w/v) DS using a mixture of cellulase (15 units/g) and pectinase (4.7 units/g) for 1 h followed by saccharifying by α-amylase (24 units/g) for 2 h and glucoamylase (0.66 units/g) for 24 h.

Pretreatment of the lignocellulosic fiber by calcium hydroxide was found to be counterproductive since it significantly decreased the subsequent susceptibility to cellulase digestion (hydrolysis) in a dose-dependent manner (Fig. 3). Certainly, alkaline pretreatment has been reported to cause solubilization, redistribution and condensation of lignin and modifications in the crystalline state of the cellulose that can lower or counteract the positive effects of lignin removal and cellulose swelling (Gregg and Saddler 1996). Likewise, it has been reported previously that the cellulose structure is changed to a form that is denser and thermodynamically more stable than the native cellulose by alkaline pretreatment (Pettersen 1984). Analysis of the calcium hydroxide pretreated hydrolysate revealed the presence of vanillin at 0.02 mg/ml, while furfural, hydroxymethylfurfural, 4- hydroxybenzaldehyde and syringaldehyde all remained below detectable limits (Table 1).

Ethanol fermentation

The starch hydrolysate, and the H_2SO_4 / cellulase treated lignocellulosic fiber hydrolysate, were each supplemented with $(NH_4)_2SO_4$ [0, 0.2, 0.4 and 0.6% (w/v)] and then fermented to ethanol by *S. cerevisiae* at 30°C, pH 4.5, without shaking for 72 h. However, neither hydrolysate required $(NH_4)_2SO_4$ supplementation as no enhanced ethanolic fermentation was observed upon the addition of $(NH_4)_2SO_4$. The maximum ethanol yields attained for the starch and the H_2SO_4/cellulase treated lignocellulosic fiber hydrolysates after 48 h of incubation were 9.9 and 11.9 g/l, respectively (Fig. 4). The experimental steps showing the glucose and ethanol yields in each step are shown in Fig. 5. Detoxification of the lignocellulosic fiber hydrolysate after

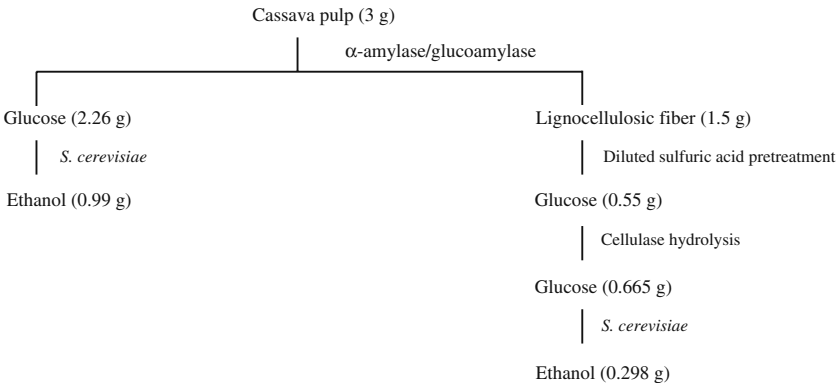

Fig. 5 Schematic summary of the experimental steps showing the glucose and ethanol yields obtained

H_2SO_4 pretreatment did not increase the ethanol yield above that found for the H_2SO_4 / cellulase fiber hydrolysates (data not shown).

However, it should be noted that carboxylic acids are important byproducts formed during the lignocellulose pretreatment step in addition to furan derivatives and water-soluble lignin degradative compounds (Millati et al. 2002).

Conclusions

Based on the method described herein, the hydrolysis of cassava pulp (3 g), which consisted of 67.8% (w/w) starch and 11.2% (w/w) lignocellulosic fiber, by α-amylase and glucoamylase yielded glucose (2.26 g) and 1.5 g (DS) of lignocellulosic fiber. Fermentation of the resultant glucose to ethanol by *S. cerevisiae* yielded 0.99 g ethanol.

Diluted sulfuric acid pretreatment and hydrolysis of the pretreated lignocellulosic fiber (1.5 g, DS) by cellulase resulted in the production of 0.665 g glucose, which upon subsequent fermentation by *S. cerevisiae* gave 0.298 g ethanol. Therefore, the total ethanol yield was 0.44 g ethanol /g glucose, and 0.429 g ethanol/g (DS) of cassava pulp.

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References

Balagopalan C, Ray RC, Sheriff JT, Rajalekshmy L (1994). Biotechnology for the value addition of waste waters and residues from cassava processing industries. In: Proceedings of the second international scientific meeting of the cassava biotechnology network. Bogor, Indonesia, 22–26 August 1994. Working document no. 150, Centro International de Agricultura Tropical (CIAT). Cali, Columbia. 690-701

Gregg D, Saddler JN (1996) A techno-economic assessment of the pretreatment and fractionation steps of a biomass-to-ethanol process. Appl Biochem Biotechnol 57-58(1):711–727

Gupta R, Sharma KK, Kuhad RC (2009) Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*- NCIM 3498. Bioresour Technol 100:1214–1220

Millati R, Niklasson C, Taherzadeh MJ (2002) Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolyzates for fermentation by *Saccharomyces cerevisiae*. Process Biochem 38:515–522

Office of the National Economic and Social Development Board (2007) Development and promotion guideline for biochemical industry. Research and Consulting Institute. Thammasart University, Thailand

Pettersen RC (1984) The chemical composition of wood (chapter 2). In: Rowell RM (ed) The chemistry of solid wood, Advances in Chemistry Series, vol. 207. American Chemical Society, Washington, DC, p 984

Somogyi M (1952) Notes on sugar determination. J Biol Chem 195:19–23

Srinorakutara T, Kaewvimol L, Saengow L (2006) Approach of cassava waste pretreatments for fuel production in Thailand. J Sci Res Chula Univ 31(1):77–84

Sriroth K, Chollakup R, Chotineeranat S, Piyachomkwan K, Oates CG (2000) Processing of cassava waste for improved biomass utilization. Bioresour Technol 71:63–69

TAPPI (1988) Test method for determination of alpha-beta and gamma-cellulose in pulp. Technical Association of Pulp and Paper Industry, 203 om-88. TAPPI, Atlanta, Georgia