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CASSAVA PULP AS A BIOFUEL FEEDSTOCK OF AN ENZYMATIC HYDROLYSIS PROCESS

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Abstract

Cassava pulp, a low cost solid byproduct of cassava starch industry, has been proposed as a high potential ethanolic fermentation substrate due to its high residual starch level, low ash content and small particle size of the lignocellulosic fibers. As the economic feasibility depends on complete degradation of the polysaccharides to fermentable glucose, the comparative hydrolytic potential of cassava pulp by six commercial enzymes were studied. Raw cassava pulp (12% w/v, particle size <320 μm) hydrolyzed by both commercial pectinolytic (**1**) and amylolytic (**2**) enzymes cocktail, yielded 70.06% DE. Hydrothermal treatment of cassava pulp enhanced its susceptibility to enzymatic cleavageas compared to non-hydrothermal treatment raw cassava pulp. Hydrothermal pretreatment has shown that a glucoamylase (**3**) was the most effective enzyme for hydrolysis process of cassava pulp at temperature 65 °C or 95 °C for 10 min and yielded approximately 86.22% and 90.18% DE, respectively. Enzymatic pretreatment increased cassava pulp vulnerability to cellulase attacks. The optimum conditions for enzymatic pretreatment of 30% (w/v) cassava pulp by a potent cellulolytic/ hemicellulolytic enzyme (**4**) was achieves at 50 °C for 3, meanwhile for liquefaction and saccharification by a thermo-stable α-amylase (**5**) was achieved at 95 °C for 1 and a glucoamylase (**3**) at 50 °C for 24 hours, respectively, yielded a reducing sugar level up to 94,1% DE. The high yield of glucose indicates the potential use of enzymatic-hydrothermally treated cassava pulp as a cheap substrate for ethanol production.

Keywords: Cassava pulp, bio-alcohol feedstock, enzymatic hydrolysis, dextrose equivalent (DE)

1. Introduction

The concept of utilizing excess biomass or waste from agricultural and agroindustrialresidues to produce energy, feeds or foods, and other useful products still in hot topic. Approximately 3.5 billion tons of agricultural residues are producedper annum in the world. Though they are rich in carbohydrate, their utilization is limited due to the low content of protein and poor digestibility [1]. Recently, bioprocessing of agro-industrial residues was found to be able to solve environmental problems associated with their disposal and has gained considerable attention because of the forthcoming scarcity of fossil fuels. The exploration of novel-efficient bioprocesses for underused biomasses is thus at the forefront of biotechnological research and industrial application.

Production of tapioca or cassava (*Manihot esculenta* Crantz) starch results in formation of 15-20% of the original processed root dry-weight basis as solid waste. This waste is well known as `pulp'. Cassava pulp is abundant and retains a high amount of starch content. Due to the high starch and moisture content (75–80% (w/w) dry weight basis), 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 principally glucose, it would not only help solve the environmental problem of cassava pulp disposal but also provides added value for cassava crops, which are typically grown by small-scale farmers in poor or developing country 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 [2]. In contrast to the above-suggested potential use, the actual current use of cassava pulp is either as a low-grade animal feed or for fertilizer. The improved

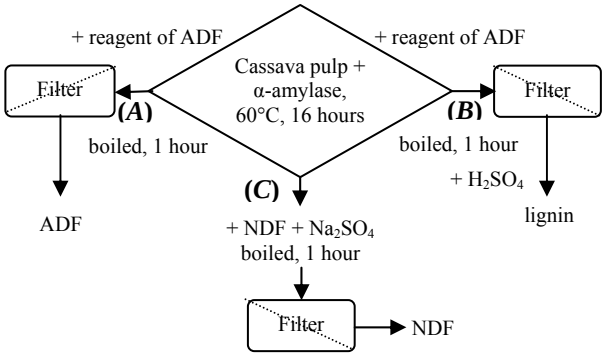


Figure 1. Analyses of Cassava Pulp; *natural detergent fiber* (NDF), *acid detergent fiber* (ADF), Cellulose = ADF – lignin, Hemicellulose = NDF – ADF

utilization of cassava pulp is, therefore, an important and necessary step towards efficient cassava root usage. Use of cassava pulp as raw material in ethanol production not only reduces waste material created from the cassava starch industry, but also lowers the cost of ethanol production [3].

So far, starch mechanical extraction in tapioca processing plant is not effective. A large amount of starch granules remains in secondary cell wall of the pulp which is mostly made of non-starch polysaccharide compounds. Enzymatically assist-depolimerization of the lignocellulosic cell wall materials with non-starch polysaccharide degrading commercial enzymes enable to liberate the starch granules from the polysaccharides entrapment and then starch degradation as well. Currently, focal point of ethanol production from feedstock of cassava pulp [4-7] is studied wether using hydrothermal process or its combination with acid pretreatment [2,8-9]. Sriroth and coworkers [4] reported that enzymatic pretreatment with mixture of cellulase and pectinase also increased releasing starches from secondary cell wall entrapment.

This study aims to reveal utilization of cassava pulp, both the starch and the non-starch polysaccharides lignocellulosic fibers by enzymatic hydrolysis to form glucose. Thereby technology certainty in mass balance of cassava pulp bioconversion into bio-alcohol will be determinable.

2. Methods

Substrate and Enzyme. Primary cassava starch (obtained by cassava root extraction) and pulp (residue material remaining afterstarch extraction) were obtained from PT. Umas Jaya Farm, Terbanggi Besar, Lampung Province. They were packaged in a frozenform and were thawed to room temperature just prior to use. Moisture contents of starch and pulp were found between 40% and 60% (wet basis), respectively. To maintain the structure of starch granule, dry pulp was prepared by a dewatering vacuum of the wet pulp (1.0 kg) at 50 °C (under cassava starch gelatinization point, 68 °C) for 24 h followed by grinding using a Grow-mill grinder NP-type (Grow Engineering Co., Ltd., Japan) at 2800 rpm and sieving through a 10–100 mesh screen (ZM-100; Retsch, Haan, Germany) to obtain a particle size of 150 μm to 2 mm and was stored in plastic bags at 4 °C prior to performing the experiment. Soluble starch, cassava starch, carboxymethyl cellulose (CMC), Birchwood xylan, and Citrous pectin were commercial products respectively used as substrate of enzyme analysis of amylase (α-amylase and glucoamylase), raw starch-digesting amylase (RSDA) cellulase, hemicellulase and pectinse, respectively. Six commercial enzyme mixtures used were cellulase of Celluclast® 1.5 L (**4**) from *Trichoderma reesei*, β-glucosidase of Cellobiase® (**2**) from *Aspergillus niger*, α-amylase of Thermamyl® 120 L (**5**), hemicellulase of Viscozyme L (**1**) from *A. aculeatus*, and glucoamylase of Dextrozyme® GA (**3**) from *A. niger* which manufactured and obtained commercially from Novo Nordisk (Bagsvaerd, Denmark), pectinase of Pectinex™ Ultra SP-L (**6**) from *A. niger*. These enzyme preparations were used without further purification.

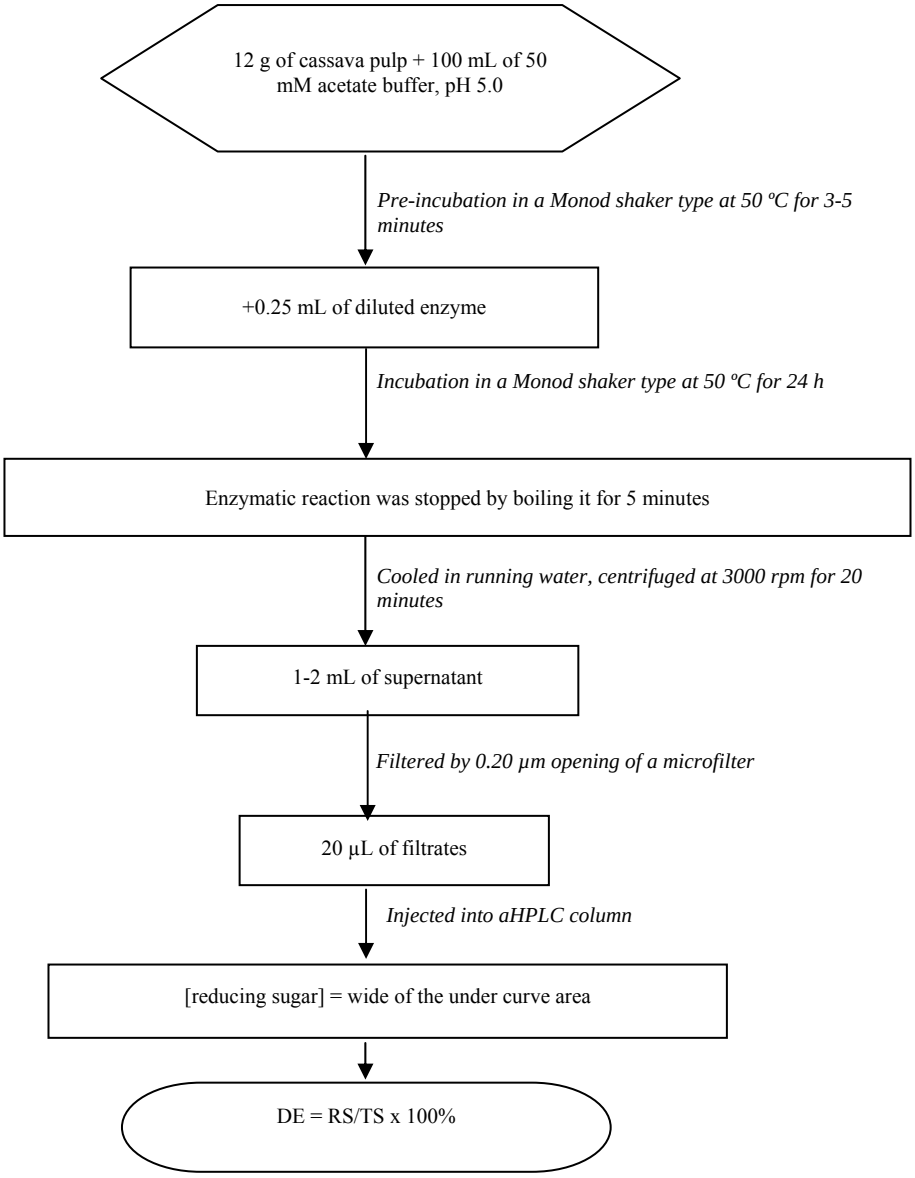
Analysis of Cassava Pulp. The proximate composition of cassava pulp was analyzed according to the AOAC standard methods [10]. Cellulose, hemicellulose and lignin were respectively determined by acid detergent fiber (ADF, [A]), natural detergent fiber (NDF, [B]) and lignin analysis (Figure 1). A mixture of cassava pulp (1.0 g) and water (600 mL) was liquefied by α-amylase solution (0.1 mL, Thermamyl 120 L, Novo Nordisk Industry Co.) at 60 °C for 16 h. The cake without starch was respectively boiled (1 h) with ADF or NDF reagents. After cooling, the mixture was separated by vacuum filtration, dehydrated, weighed the filter crucibles and determined gravimetrically (Sartorius AG, Model BP 211D, Göttingen, Germany). The pellets of the ADF-treatment were diluted with 2 N sulfuric acid to give an acid-insoluble residue that was dried and weighed as lignin. The content of protein in the pulp (total N by Kjeldahl method x 6.29), fat (by Soxhlet with petroleum ether extraction) and ash (incineration at 600 °C for 3 h) were quantified as described by AOAC methods [10].

Enzymatic hydrolysis of cassava pulp. Rate of cassava pulp enzymatic hydrolysis was expressed by accumulation of reducing sugar in the hydrolysate (Figure 2). The pretreated pulp slurries were enzymatically hydrolyzed to determine the maximum obtainable sugar. The pH of the slurries was pre-adjusted to 5.0 with sodium hydroxide, and 3 M sodium acetate buffer at pH 5.0 was added at a final concentration of 50 mM. The 100 mL reactions contained 12% cassava pulp in 50 mM sodium acetate

buffer (pH 5.0) with appropriate amount of commercial enzyme mixture consisting of different combinations of cellulase, pectinase, hemicellulase, and α-glucoamylase was incubated at 50 °C for 24 h with rotary shaking at 200 rpm, stopped by heating in boiled water for 5 min, and cooled by running water.

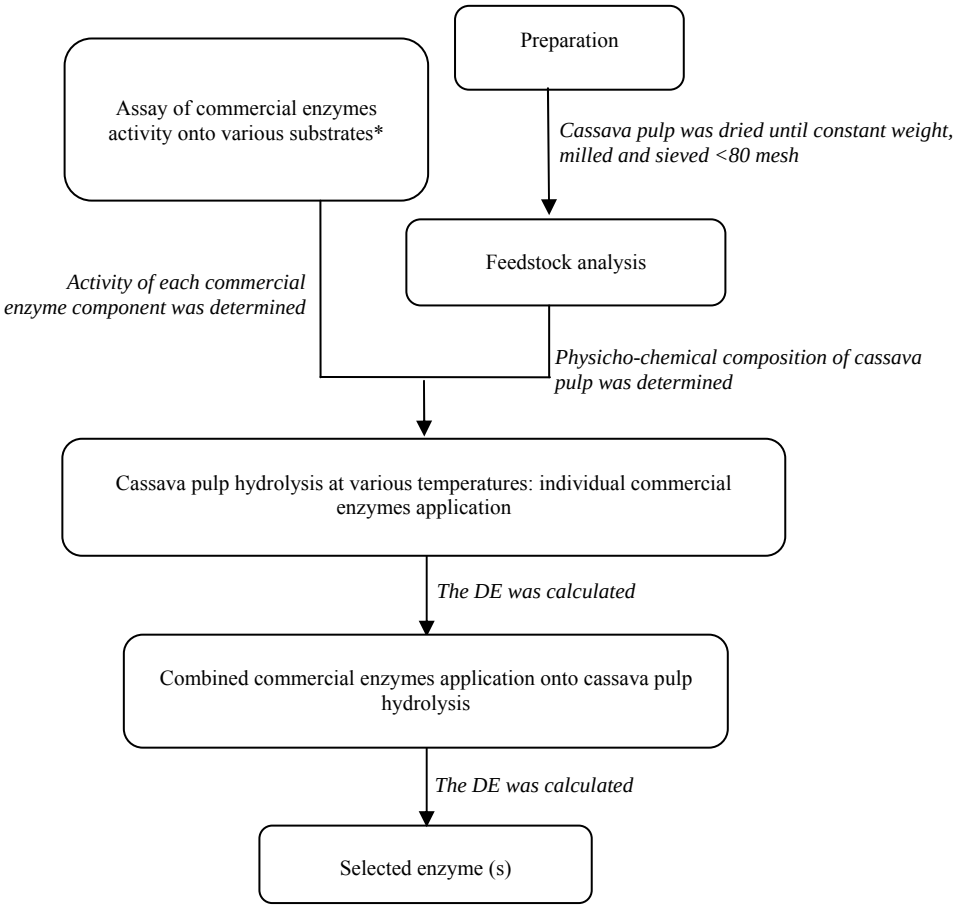
The supernatant, referred to as the starch hydrolysate, was harvested by centrifugation at 3,000 x *g* for 20 min to precipitate un-dissolved substrate and assayed for reducing sugar. The sugar concentration was determined by high-performance liquid chromatography (HPLC, ShimidzuCBM 10A, Japan) equipped with a 4.6 mm x 150 mm Shimpak SCR 101 P carbohydrate analysis column and a RID 10A diferensial refractive index detector. The mobile phase was deionized water at flow rate of 1.0 mL/min, and column temperature was set at 60 °C. Reducing sugar concentration of cassava pulp hydrolysates was basically determined on chromatogram width of the under curve area. Dextrose equivalent (DE) that expresses a hydrolysis conversion rate was calculated as a reducing sugar (as glucose) ratio towards total sugar of the cassava pulp.

Flowchart of the research. Overall researches were progressively conducted as a represented flow-chart at Figure 3.



DE = dextrose equivalent, RS = reducing sugar, TS = total sugar

Figure2. Hydrolyses of Cassava Pulp



*CMC, xylan, citrous pectin or *soluble starch,* DE = *dextrose equivalent*

Figure 3. Flowchart of Research and Analyses of the Respective Stages

3. Results and Discussion

Physico-chemical composition of cassava pulp. Carbohydrate composition of cassava pulp was analyzed, and summarized in Table 1. Total glucose derived from starch, cellulose and soluble portion occupied approximately 94.7% of dry matter. Starch was the main polysaccharide in cassava pulp, comprising 65.6±1.6% and 39.4% of dry and wet matters, respectively. Non-starch polysaccharide contains fibrous materials, cellulose, hemicelluloses, pectin, protein and lignin, of 20.1%, 8.1%, 2.8%, 7.0%, 3.1%, 2.2%, respectively. A high starch constituent within a waste reflects an efficiency level of starch extraction process from a tapioca factory. The cassava pulp was dried below the starting gelatinization temperature of cassava starch to preserve the physical characteristics of the starch granules [11], hemicelluloses [12] and lignin [13]. Fibers content 20.1% of cassava pulp which is reported in this study was lower than previous reported by Kosugi [8], Rattanachomsri [7], Sriroth [4], Thongchul [14] which were 29%, 23.0%, 27.75% and 35.9%, respectively. Cellulose (8.1±1.1%) and hemicellulose (2.8±0.1%) contents were lower than those reported previously [7] but lignin content (2.2±0.2%) was comparable to those reported previously [7,8]. Glucans were the major polysaccharide [8] while major monosaccharide of the cassava a pulp analysis was galactose (78%), and successively followed by arabinose (10.7%), rhamnose (6.2%), glucose (5.1%), xylose (2.5%), fuccose (0.7%) [15]. Arabinose, xylose, rhamnose, galactose and fuccose composed approximately 7.16% of dry matter, which could be mainly derived from hemicelluloses (2.8±0.1%) and pectin (12.4%) fractions.

Different from Kosugi [8], galactose content in this study was 7–111 times higher than arabinose, rhamnose, glucose, xylose, and fuccose contents. This supported that hemicelluloses and pectin of cassava pulp is rich in galactan [15]. In this study, the tapioca industry probably employed either higher starch or lower fiber portion of an earlier-matured cassava cultivar. As a main waste portion of cassava pulp, fibers will enhance by the increase of age. Table 1 presented that portion of pectin substance (±7.0%), a primary

Table 1. Physico-chemical Composition of Cassava Pulp (g/100 g Dry Wheight)[a]

Components	Sriroth et al. [4][b]	Kosugi et al. [8]	Rattanachomsri et al. [7]	Akaracharanya et al. [9]	Thongchul et al. [14]	In this study
Starch	68.8±4.0	60.6	60.1±0.09	67.8	50.0	65.6±1.6
Reducing sugar[d]		4.7	-	-	-	-
Non-starch polysaccharides:						
Fiber	27.8±0.2	28.1	23.0	11.2	35.9	20.1
- Glucans	-	19.1	-	-	-	-
- Xylans	-	4.2	-	-	-	-
- Arabinans	-	1.4	-	-	-	-
- Galctans	-	0.5	-	-	-	-
- Mannans	-	0.7	-	-	-	-
Cellulose[e]	-	-	15.6	-	-	8.1±1.1
Hemicellulose[f]	-	-	4.6	-	-	2.8±0.1
Pectin[g]	-	-	-	-	-	7.0
Lignin	-	2.2	2.8±0.06	-	-	2.2±0.2
Protein	1.6±0.03	2.49[h]	-	-	5.3	3.1±0.0
Fats	-	-	-	-	0.1	0.2±0.0
Ash	1.7±0.01	-	-	-	-	5.7±0.1
Mischeleneous	-	0.9	-	-	-	-
Total	-	94.7	-	-	-	94.7
pH	4.99	-	-	-	-	5.00

[a]dry weight basis
[b]mean ± standard deviasion of two replicates
[c]mean ± standard deviasion of three replicates
[d]free sugars as glucose
[e] Acid detergent fiber (ADF) - lignin.
[f]Neutral detergent fiber (NDF) - ADF
[g] mathematics calculation
[h] after times to factor of 6.29 (ratio of cassava pulp protein to the total nitrogen (N_{total}) by method of Kjeldahl analyses)

and middle lamella cell wall component of cassava pulp, was presumably comparable to fresh cassava tuber pectin 10.38% [16]. Cassava had the lowest pectin fraction (17.8%) as well as galacturonic acid content (17%) within its cell wall materials (CWM) at only 7.3% [13] among tuber crops but the highest cellulose fraction (48.2%). Pectin fraction had a slightly higher than hemicelluloses fraction and both highly influence towards both slurry viscosity and turbidity. A high viscosity will complicate the process handling and lowering both starch hydrolysis rate to fermentable sugars [17] and the fermentation efficiency [18]. Pectin and other fiber components (cellulose and hemicelulose) can increase the cassava pulp capacity in both water absorbance and grip. Therefore the moisture ranged between 60% and 70%. Based on cassava pulp composition (Table 1), enzymes that can degrade non-starch polysaccharides components such as cellulose, hemicellulose and pectin are needed. Therefore, it was necessary to select both single and commercial enzyme combination to make the hydrolysis process goes optimally. According to the available amount of fermentable sugars (soluble sugars and starch), 1 kg of fresh cassava pulp would yield approximately 195 g or 284 mL of ethanol, if complete conversion of fermentable sugars to ethanol is accomplished. This number is greater than potential yield of potato pulp hydrolysis: 126 g or 160 mL of ethanol [19]. Nitrogen elements that express a protein content of the cassava pulp were very poor (0.32–3.13%). Meanwhile, the ash substances were much lower than sugarcane bagasse; therefore enzymatic degradation of cassava pulp is quite easy too. This nutritional analysis is also very useful in improvement of cassava pulp hydrolysis methodology.

Profie of commercial enzymes. A commercial enzyme poses the multiple enzyme activities [20]. To detect any enzyme within a commercial enzyme, a series of experiments were carried out by using various substrates which were likely similar to cassava pulp components. Table 2 shows hydrolytic enzymes against various substrates.

Tabel 2. Profile of Commercial Enzymes Role Based on Its Activity Onto Substrates

Code	Brand	Enzyme activity (U/mL) onto below substrates				The major role
		Carboxymethyl cellulose (CMC)[1]	Xylan[2]	Citrous pectin[3]	*Soluble starch*[4]	
2	Cellobiase®	3.45	7.52	4.51	169.67	Amylolytic
4	Celluclast 1.5 L	21.58	21.46	12.67	1.68	Cellulolytic/Hemicellulolytic
3	Dextrozyme®GA	5.14	3.07	18.08	165.73	Amylolytic
6	Pectinex™ Ultra SP-L	7.69	6.24	48.33	35.19	Pectinolytic
5	Thermamyl® 120 L	10.09	14.51	9.75	78.78	Amylolytic
1	Viscozyme® L	8.22	16.50	47.60	55.33	Amylolytic/ Pectinolytic

[1] expressed cellulases activity
[2] expressed hemicellulases activity
[3] expressed pectinases activity
[4] expressed amylases activity

Polysaccharide degrading activities were analyzed based on the amount of liberated reducing sugars using Somogy-Nelson method [21]. The 2.5 mL reactions contained the appropriate dilution of commercial enzyme in 50 mM sodium acetate buffer, pH 5.0 and 1% of the corresponding substrate: pectin from citrus for pectinase activity, birchwood xylan for hemicellulase activity, carboxymethyl cellulose for CMCase activity, and soluble starch for α-amylase activity. The reaction was incubated in a reciprocal water bath incubator shaker at 50 °C and 80 rpm. After 1 h, the supernatant from 1.5 mL of hydrolysate was separated by centrifugation at 3,000 x *g* for 10 min and was assayed for the reducing sugars. The amount of reducing sugars was determined from the absorbance measurements at 660 nm and interpolated from a standard curve of the corresponding sugar. One unit was defined as the amount of enzyme that produced 1 mmole of reducing sugar from substrates by 1 mL of enzyme per minute.

This experiment elucidated that **2**, **3**, and **5** were dominant in starch hydrolysis with activity of 169.67 U/mL, 165.73 U/mL and 78.78 U/mL, respectively. **6** has been proved as pectinolytic enzyme [3] with the activity of 48.33 U/mL similar to **1** (47.33 U/mL), a hemicellulase with 112 fungal β-glucanase unit (FBG)/ mL [20], even though its amylolytic activity was significant enough (55.33 U/mL). Buchert and coworkers [22] also proved that **6** didn't have overall cellulolytic activity or filter paper activity while **1** (a multienzyme complex with cellulase, hemicellulase, arabanase, xylanase and β-glucanase activities) from *Aspergillus niger* has 100 β-BG/g. **4** is the only commercial enzyme which is more active in non-starch carbohydrate decomposition with the activities of cellulase, hemicellulase and pectinase are 21.6 U/mL (38% of the total activity), 21,5 U/mL (37% of the total activity) and 12,7 U/mL (22% of the total activity), respectively [23].

Application of multi-enzyme in raw cassava pulp hydrolyses. Analysis of a decomposing cassava pulp was carried out by comparing its dextrose equivalent (DE). The DE is ratio of reducing sugar accumulation within an enzymatic hydrolysate to its total sugars of the substrate (cassava pulp). High fiber content within cassava pulp plays a significant role in retarding starch extraction as the fibrous network holds starch granules together. In plant cells, cell walls consist of microstructures composed of cellulose embedded in a polysaccharide and protein matrix surrounded by an outer layer composed mainly of pectic material [24]. Starch granules contained in this complex polymer matrix are difficult to liberate. Therefore, to improve starch extraction from the pulp, this complex matrix must be disrupted so that starch granules can be liberated. The disruption of the fibrous matrix can be achieved either by enzymatic or physical method. Another alternative to increase the efficiency of starch recovery from cassava pulp is by applying the cellulase/pectinase mixture. Cellulases and pectinases destroy the structural integrity of the matrix which is responsible for trapping starch granules; the outcome of this action is to expose the granules, and increase. Enzymatic treatment was the main concern to provide better starch recovery and feasibility in industry. Enzymatic saccharification of cassava pulp is distinct from raw cassava starch or cassava root homogenate because of the trapped nature of raw starch granules and higher non-starch polysaccharide content in pulp. The cooperative action of an array of polysaccharide hydrolyzing enzymes is thus essential for efficient degradation of this substrate. For the first stage, multi-enzymatic activities involved in non-thermal cassava pulp saccharification were investigated systematically using different commercial enzyme combinations (Table 3).

Enzymatic hydrolysis by individual non-starch polysaccharide hydrolyzing enzymes (**4** and **6**) at high

enzyme loading released relatively low levels of reducing sugar (Table 4), which were derived from decomposition of the cell wall components. According to Sriroth [4] the combined action of cellulase (**4**) and pectinase (**6**) yielded more reducing sugar owing to the efficient hydrolysis of the non-starch fibrous structure and the cementing pectin containing compounds. On the other hand, addition of commercial β-glucanase /hemicellulase (such as **1**) to the enzyme mix did not appreciably increase the fermentable sugar yield [7]. This might be due to the presence of high hemicellulase side activity of the commercial cellulase. In contrast, addition of a commercial β-glucosidase (such as **2**) led to a marked increase in reducing sugar yield. The higher yield of reducing sugar caused by the presence of strong raw starch-hydrolyzing side activity of the commercial enzyme preparation, in addition to the possible direct effect from the β-glucosidase activity on cellulose-derived substrates. Raw starch digesting amylolytic enzyme (RSDAE) attacks and degrades a starch granule surface from its end of non-reducing macromolecules [25], but it must be previously released from non-starch polysaccharides constrain structure. The structure can be decomposed by cellulase, hemicellulase and pectinase enzymes. Therefore the slurry of the raw cassava pulp would be liquefied. Hydrolysis of 12% (w/v) raw cassava pulp (particle size <320 μm or 80 mesh) by commercial enzyme combination of **1** and **2** at 50 °C for 24 hours yielded 70.1% DE (Table 4). This combination consists of 0.724 U/g cellulase, 1.458 U/g hemicellulase, 4.109 U/g pectinase and 5.702 U/g amylase. The DE of this study was higher than those were achieved by Sriroth [4] and Thongchul [14] but lower than those were achieved by Rattanachomsri [7]. Addition of mixture of different enzymes (2 U/g cellulase, 1.6 U/g hemicellulase, 4.9 U/g pectinase and 0.7 U/g cassava pulp of RSDAE preparation of *Aspergillus niger* BCC17849) could hydrolize of 4% (w/v) raw cassava pulp in 50 mM Na-asetat buffer (pH 5.0) at 40 °C for 48 hours and shaked at 200 rpm, regenerated 91.2% DE of hexoses and 97.2% of pentoses, respectively. Even though they obtained higher DE, the concentration of cassava pulp substrate was only one-third of our study (4 g/100 mL vs 12 g/100 mL). On the other hand, saccharification of the substrate by excess glucoamylase only led to a relatively low reducing sugar yield [7]. Further reduction of glucoamylase loading (5,702 U/g of **1** + **2** to 5,680 U/g of **3** + **1**) resulted in decrease significantly on the saccharification yield (70.06% DE to 60.13% DE) which was compared to 4,747 U/g of **1** (64.02% DE see Table 4). In other experiment, increasing glucoamylase did not show any effect on reducing sugar yield (39.39% DE by 7,692 U/g of **3** + **5**), suggesting excessive use of glucoamylase under these experimental conditions. It should be noted that the relative glucoamylase activity on cassava pulp was approximately 32% of that on the extracted raw cassava starch. The activity was measured based on the released reducing sugar yield using the same RSDAE unit of glucoamylase under the same conditions [7]. This indicated the lower accessibility of the enzyme to the trapped starch granules in cassava pulp.

Table.3. Preliminary Assessment

Enzyme codes	Hydrolysate aaccumulations (mg of reducing sugar/mL/minute)		
	Raw[1]	65 °C[2]	90 °C[2]
2	14.06	84.65	132.34
4	12.99	13.07	10.56
3	59.41	82.50	145.60
6	3.01	17.04	30.17
5	67.97	79.33	85.79
1	20.38	52.92	52.65

[1] Non-hydrothermal pretreatment
[2] Hydrothermal pretreatment for 10 minutes
Dosage per gram substrate (cassava pulp) of **2** (Cellobiase®), **3** (Dextrozyme®GA), **5** (Thermamyl®120L), **4** (Celluclast 1.5 L), **6** (Pectinex™ Ultra SP-L), dan **1** (Viscozyme®L) were respectively 0.00563 mL, 0.00563 mL, 0.0858 mL, 0.0858 mL, 0.0858 mL, dan 0.0858 mL. Reducing sugar concentration of cassava pulp hydrolysates was determined by a HPLC method.

As suggested from the substrate's chemical components and structure, the combination of the multiple non-starch polysaccharide degrading enzymes and the enzymes with strong RSDAE activity led to a respective increase in fermentable sugar yield (70.06% DE of **1** + **2**). This suggested the cooperative action of multi-non starch polysaccharide hydrolyzing activities in degradation of the fibrous cell wall structure together with the amylolytic RSDAE activity acting on the accessible released starch granules. The results thus indicate that efficient saccharification of cassava pulp with no pre-gelatinization is possible by the combined actions of multiple non-starch polysaccharide hydrolyzing enzymes and RSDAE.

Hydrothermal and enzymatic pretreatment of cassava pulp hydrolysis. Conventionally ethanol production from a starchy material had been preceded by a hydrothermal pretreatment that causes its viscosity increase 20 times thicker than its slurry and hence more difficult to be pumped or stirred. The main substances which are responsible for the increasing viscosity are pectin and hemicelluloses [12]. To liquefy and to decompose them into a simple sugar (saccharification), the starchy materials had to be given appropriate enzymatic treatments of α-amylase and glucoamylase, respectively. **5** and **3** were starch decomposing commercial enzymes with the dominant role as α-amylase and glucoamylase, respectively (Table 2). Homogeneity of the gelatinized cassava pulp as a

Table 4. Cassava Pulp Hydrolysis at Various Hydrothermal Pretreatment Temperatures

Single or enzyme combination	Prediction of a commercial enzyme component activity (U/g substrate)				Dextrose equivalent (DE)		
	Cellulases	Hemicellulases	Pectinases	Amylases	Raw[1]	65 °C[2]	90 °C[2]
3	0.029	0.017	0.102	0.933	36.35	86.22	90.18
3 + 6	0.689	0.552	4.246	3.952	53.26	73.36	88.12
3 + 1	0.734	1.433	4.186	5.680	60.13	79.82	80.69
3 + 5	0.895	1.262	0.939	7.692	39.39	62.90	75.41
3 + 4	1.881	1.858	1.189	1.077	38.86	79.14	72.64
2 + 1	0.724	1.458	4.109	5.702	70.06	79.23	76.21
2 + 4	1.871	1.883	1.112	1.099	39.89	69.79	71.32
2 + 6	0.679	0.577	4.169	3.974	54.91	75.24	71.40
2 + 5	0.885	1.287	0.862	7.714	33.56	57.45	75.62
2	0.019	0.042	0.025	0.955	21.66	65.69	70.20
4 + 5	2.718	3.086	1.924	6.903	18.70	17.80	17.20
5	0.866	1.245	0.837	6.759	12.25	13.39	10.71
1	0.705	1.416	4.084	4.747	64.02	70.14	71.77
6	0.660	0.535	4.144	3.019	44.65	77.22	62.50

[1] Non-hydrothermal pretreatment
[2] hydrothermal pretreatment for 10 minutes
DE (dextrose equivalent) is ratio of reducing sugar accumulation within an enzymatic hydrolysate to its total sugars of the substrate.

substrate of enzymatic reaction was much better than raw starch suspension in water. Starches of cassava pulp initiate to gelatinize at the temperature of 68 ºC [26], a small portion of water molecule accomplished to entering the granules thus they were swollen. At moderate temperature in hydrothermal treatment at 65 ºC for 10 minutes, and hydrolysis using **3** at 50 °C for 24 hours, a higher DE (86.22%) was achieved (Table 4). Meanwhile, hydrolysis using **3** at 50 °C for 24 hours and at elevated hydrothermal (90 ºC, 10 minutes) was reached up to 90.18% DE. It verifies that **3** not only has amylolytic activity but also sufficient in both fiber and pectin decomposing activity therefore it was able to well-decompose both cassava pulp cell wall structure and the starch captured inside. Either higher temperature hydrothermal pretreatment or lower concentration substrates made more homogeneous cassava pulp suspensions. Therefore larger DEs were not difficult to be achieved. By lower substrate concentration (5%, w/v) and higher hydrothermal pretreatment (140 ºC, 1 hour), Kosugi [8] obtained 91% DE from hydrolysis of cassava pulp by adding 3 U/ g cellulase (50 °C, 72 hours) and 300 U/g α-amylase + 100 U/g glucoamylase (50 °C, 48 hours). They obtained a slight higher DE of 91% than our study of 90.18%. It was caused by applying both the higher hydrothermal pretreatment temperature (140 ºC/L h vs. 90 ºC/10 minutes) and the lower concentration substrate (5% w/v vs. 12% w/v) therefore homogeneity of the gelatinized cassava pulp as a substrate was tremendous better.

Table 5. Enzymatic Pretreatment

Experiments	Enzymatic pretreatment	Application	Dextrose equivalent (%)
A	**6**		79.4
B	**1**	**5 + 3**	73.2
C	**4**		94.1

A-B. Cassava pulp suspension (12%, w/v) within 50 mM of acetate buffer (pH 5.0) were enzymatic pretreated by adding 0,0858 mLof **6** (A) or **1** (B) and incubated at 50 °C for 3 hours as an enzymatic pretreatment. Reducing sugar of the supernatant was analyzed by HPLC [23].

C. Cassava pulp suspension was enzymatic pretreated by **4**, liquefied and saccharified by respective **5** and **3**. Then the reducing sugar was determined according to Somogyi-Nelson [21].

Starch granules were greatly gelatinized by consequence of hydrothermal treatment (90 ºC), the starch molecules were saturated by water thus hydrolytic enzymes such as amylolytic, cellulolytic, hemicellulolytic and pectinolytic enzymes spatially attacked them. Application of commercial enzyme prior to hydrothermal pretreatment/liquification can be assumed as a part of the pretreatment (enzymatic pretreatment).

Sriroth [4] considered that cassava pulp hydrolysis using combination of cellulase and pectinase enzymes

are applied to release the starch granules from the cell wall trap easily. As the consequence, decomposition of the cell wall also decreases the slurry viscosity by 30% [27]. Reduction of the slurry viscosity was supposedly more related to activity of non-starch polysaccharide hydrolyzing enzymes. Combination of cellulase and pectinase enzymes proved to boost both hydrolysis efficiency of both fibers structure and pectin molecules [4]. It also produced a slight reducing sugar from the cell wall component [7]. The increase value of DE was more likely related to amylases and β-glucosidase activity that release glucose from starch and cellulose/ cellulose intermediates, respectively. Enzymatic pretreatment of cassava pulp using **6**, **1** or **4** enzymes not only increases DE but also reduce the broth viscosity as seen in Table 5. Superior DE in this experiment was achieved by addition of **4** (50 °C, 180 minutes) as an enzymatic pre-treatment prior to hydrothermal treatment (90 °C, 10 minutes) and enzymatic liquefaction and saccharification by **5** (90 ºC, 60 minutes) and **3** (50 ºC, 24 h) respectively [23]. Application of **4** as the enzymatic pretreatment aimed to liquefy the slurry hence the broth transfer to saccharification compartment was easier and contamination would reduced.

4. Conclusion

Cassava pulp proposed as a high potential ethanolic fermentation substrate due to its high residual starch level, small particle size of the lignocellulosic fibers, low ash content, nearly similar starch granule size and property to cereals which are entirely easier to be enzymatic hydrolysis. Cassava pulp cell wall materials pose the lowest pectin which highly influence of the slurry viscosity and turbidity but the highest cellulose among the tuber pulps. Acid hydrolysis was substituted by hydrothermal/enzymatic pretreatment and enzymatic hydrolysis, due to the appearance of ash substances which hindered the enzyme activity. Raw cassava pulp (12% w/v, particle size <320 μm) hydrolysis by both commercial pectinolytic (**1**) and amylolytic (**2**) enzymes cocktail, DE 70.06% was realizable. Hydrothermal treatment at 65 ºC or 90 ºC for 10 min using glucoamylase (**3**) produced higher DE, 86.22% or 90.18%, respectively. Optimization of enzymatic pretreatment by a potent cellulolytic/hemicellulolytic enzyme (**4**, 3 h) prior to hydrothermal treatment (90 °C, 10 min) and enzymatic liquefaction and saccharification by respective thermo-stable α-amylase (**5**, 90 ºC, 1 h) and glucoamylase (**3**, 50 ºC, 24 h), was achieved highest DE (94.1%).

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