Simultaneous saccharification and viscosity reduction of cassava pulp using a multi-component starch- and cell-wall degrading enzyme for bioethanol production

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Abstract In this study, an efficient ethanol production process using simultaneous saccharification and viscosity reduction of raw cassava pulp with no prior high temperature pre-gelatinization/liquefaction step was developed using a crude starch- and cell wall-degrading enzyme preparation from *Aspergillus aculeatus* BCC17849. Proteomic analysis revealed that the enzyme comprised a complex mixture of endo- and exo-acting amylases, cellulases, xylanases, and pectinases belonging to various glycosyl hydrolase families. Enzymatic hydrolysis efficiency was dependent on the initial solid loading in the reaction. Reduction in mixture viscosity was observed with a rapid decrease in complex viscosity from 3785 to 0.45 Pa s with the enzyme dosage of 2.19 mg/g on a dried weight basis within the first 2 h, which resulted from partial destruction of the plant cell wall fiber and degradation of the released starch granules by the enzymes as shown by scanning electron microscopy. Saccharification of cassava pulp at an initial solid of 16% (w/v) in a bench-scale bioreactor resulted in 736.4 mg glucose/g, which is equivalent to 82.92% glucose yield based on the total

starch and glucan in the substrate, after 96 h at 40 °C. Simultaneous saccharification and fermentation of cassava pulp by *Saccharomyces cerevisiae* with the uncooked enzymatic process led to a final ethanol concentration of 6.98% w/v, equivalent to 96.7% theoretical yield based on the total starch and cellulose content. The results demonstrated potential of the enzyme for low-energy processing of cassava pulp in biofuel industry.

Keywords *Aspergillus aculeatus* · Bioethanol · Glycosyl hydrolases · Saccharification · Viscosity

Introduction

Conversion of agricultural wastes to biofuels, biochemicals, and biomaterials is a promising strategy that could help alleviating our dependence on non-renewable fossil resource while, at the same time, solving environmental problems related to their disposal (Menon and Rao 2012). Cassava (*Manihot esculanta*) is one of the most economically important crops in tropical and subtropical regions, owing to high crop productivity as well as high starch and low ash contents with easily hydrolyzable nature compared to most grains and other tuber crops (El-Sharkawy 2004). Processing of cassava in starch industry generates cassava pulp as the major by-product. The pulp contains approximately 60% residual starch by weight while the rest comprises cell wall polysaccharides, making it a unique starting material for biorefineries compared to most other second-generation cellulosic substrates. It is generally used for low-value products such as animal feed or discarded as waste; however, its potential for conversion to a range of commodity fermentation products has been recently emerged (Pandey et al. 2000).

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Bioprocessing of starch-based materials by polysaccharide-degrading enzymes in combination with microbial conversion is an energy-intensive process. In the conventional processing, a high-temperature pre-gelatinization/liquefaction step in the presence of a thermostable α -amylase is required, followed by maltodextrin saccharification with glucoamylase (Sriroth et al. 2000a). The hydrolysate can then be fermented to produce ethanol and organic acids such as fumaric acid and lactic acid (Carta et al. 1999). Direct saccharification of starch-based substrates without pre-gelatinization using raw starch-degrading (RSD) amylolytic enzymes of fungal origins e.g. *Aspergillus* spp., *Rhizopus* spp., and *Penicillium* spp. can provide substantial energy savings compared with the conventional process (Norouzian et al. 2006; Lin et al. 2011; Poonsrisawat et al. 2014). The ease of this native starch hydrolysis can vary greatly, depending on their botanical origins which determine morphology (size, surface features, peripheral pores and channels) and crystallinity of the starch granules (Lehmann and Robin 2007). Cassava starch granules are generally more resistant to enzymatic hydrolysis than cereal starches (Piyachomkwan et al. 2007). The starch granules are entrapped in cell wall structure composed of a complex lignocellulosic matrix containing cellulose and hemicelluloses with a relatively high pectin content in dicotyledonous plants (Sriroth et al. 2000a; Sørensen et al. 2000). This hinders their efficient degradation (Nitayavardhana et al. 2008) and results in high viscosity of the fermentation mixture due to the swelling properties of the cell wall polysaccharides. Efficient non-thermal enzymatic hydrolysis of dried cassava pulp with no prior high temperature pre-gelatinization step using various combinations of commercial cellulolytic, hemicellulolytic, pectinolytic, and amylolytic enzymes and a crude multi-component enzyme from *Aspergillus aculeatus* has been reported (Rattanachomsri et al. 2009). The use of a crude enzyme preparation with multiple cell wall-degrading activities from this fungal strain for viscosity reduction of different cassava products in very high gravity (VHG) fermentation has also been recently demonstrated (Poonsrisawat et al. 2014).

In this study, a process involving direct hydrolysis of raw cassava pulp without a pre-gelatinization/liquefaction step with concurrent viscosity reduction of the fermentation mixture was developed using the multi-component starch and cell wall-degrading enzyme from *A. aculeatus*. The fungal secretome was analyzed by proteomic analysis and revealed a complex mixture of glycosyl hydrolases within various families targeting on decomposition of starch and lignocelluloses. The enzyme was applied for non-thermal simultaneous saccharification and fermentation (SSF) of cassava pulp to ethanol in a bench-scale bioreactor with a high ethanol titer. The work provides an

alternative process for efficient conversion of cassava pulp to biofuels and other value-added fermentation products with improved energy efficiency.

Materials and methods

Substrate

The dried cassava pulp was obtained from Chonchareon Ltd., Chonburi, Thailand. The chemical compositions of cassava products were analyzed for moisture, protein, ash, and sand content using a standard method (AOAC 1990). Starch content was determined using the polarimetric method, according to the standard method of The Commission of the European Communities (1999) and Thai Industrial Standard for Tapioca Flour/Starch (1978). Neutral and acid detergent fiber (NDF and ADF) and lignin were analyzed following the standard AOAC method (2002) (Method 992.16 and Method 973.18). The fresh cassava pulp contained $78.09 \pm 0.45\%$ moisture. It was composed mainly of starch ($62.87 \pm 0.03\%$) and fibrous material containing cellulose (17.13%) and hemicellulose (5.40%), which are sources of fermentable sugars, in addition to other components e.g., protein ($1.50 \pm 0.04\%$), lignin ($3.43 \pm 0.16\%$), and ash ($1.84 \pm 0.01\%$). The pulp was dried at 50 °C which was below the gelatinization temperature of cassava starch ($T_0 = 58.5$ °C) to preserve the physical characteristics of the starch granules (Gomand et al. 2010).

Microorganisms and inoculum preparation

Aspergillus aculeatus BCC17849 was obtained from BIOTEC Culture Collection, (<http://www.biotec.or.th/bcc>) where it is stored in deep freezer and liquid nitrogen according to the standard procedure for long-term storage. The fresh strain was maintained on potato dextrose agar (PDA). Thermotolerant yeast *S. cerevisiae* (Thermosacc® Dry yeasts, Lallemand Ethanol Technology, Milwaukee, WI) was used for ethanol production. Yeast inoculum was grown in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose. The culture was incubated at 30 °C for 24 h with rotary shaking at 200 rpm. The yeast cells were then harvested and washed with sterile 0.9% w/v saline solution to remove glucose residue in the culture medium. All the media were autoclaved at 121 °C for 15 min prior to use.

Enzyme production by solid state fermentation

The multi-component enzyme was prepared according to the previously reported method by Poonsrisawat et al.

(2014). *A. aculeatus* grown on rice grains was inoculated in liquid spore production medium (2% peptone, 2% yeast extract, 1% KH_2PO_4 , and 1% glucose) at the ratio of 1 g rice: 1 mL medium. The culture was incubated at 30 °C for 3 days. The fungal spores were resuspended in 0.1% Tween 80 and the suspension was used as the starter culture. The starter culture was inoculated into the solid-state production medium (20 kg on dried weight basis) containing 3.5:1.5 (w/w) of dried cassava pulp: soybean meal supplemented with 20 g/kg yeast extract, 20 g/kg peptone, 250 mL/kg salt solution (40 g/L KH_2PO_4 , 90 g/L Na_2HPO_4 , and 6 g/L KCl), and 10 mL/kg trace element solution (14.3 g/L $\text{ZnSO}_4\cdot\text{H}_2\text{O}$, 0.5 g/L $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$, 2.5 g/L $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, and 13.8 g/L $\text{FeSO}_4\cdot\text{H}_2\text{O}$). The moisture content was adjusted to 70%. The culture was grown at 30 °C for 5 days in a tray reactor system. The enzyme was extracted by adding 50 mM sodium acetate buffer, pH 5.5 containing 0.1% Tween 80 to the solid-state culture with the solid:liquid ratio of 1:4. The mixture was incubated at 4 °C overnight. The crude enzyme mixture was clarified by centrifugation, filtered sequentially through 0.45 and 0.2 μm nitrocellulose membranes (Sartorius, Goettingen, Germany) and then concentrated 15-fold by tangential flow filtration on a Minimate tangential flow filtration system using a 30 kDa MWCO membrane (Pall, Port Washington, NY). The enzyme was stored at 4 °C for subsequent experiments.

Enzyme activity assay

Polysaccharide-degrading activities were analyzed based on the amount of liberated reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The 1 mL reactions contained an appropriate dilution of enzyme in 50 mM sodium acetate buffer, pH 5.0 and 1% (w/v) of the corresponding substrate: pectin from citrus for the determination of pectinase activity, birchwood xylan for the determination of hemicellulase activity, carboxymethyl cellulose for determination of CMCase activity, β -glucan for the determination of β -glucanase activity, locust bean gum for determination of mannanase activity, soluble starch for determination of α -amylase activity, or raw cassava starch for the determination of RSD activity. The reaction was incubated at 40 °C for 30 min. Cellulase expressed as filter paper activity (FPU) was analyzed in a 1 mL reaction using Whatman number 1 filter paper (size $1 \times 6 \text{ cm}^2$) as substrate and incubated at 40 °C for 60 min (Ghose 1987). The amount of released reducing sugars was determined from the absorbance measurements at 540 nm and interpolated from a standard curve of the corresponding sugar. β -Glucosidase and β -xylosidase activities were determined using *p*-nitrophenyl- β -D-glucopyranoside (pNPG) and *p*-nitrophenyl- β -D-xylopyranoside (pNPX) as

substrate, respectively (Ghose and Bisaria 1987). One international unit (IU) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar or *p*-nitrophenolate in 1 min under the assay condition.

Proteomic analysis

Proteomic analysis of the crude enzyme was performed according to Wongwilaiwalin et al. (2010). The enzyme preparation was applied to a 10% polyacrylamide gel and subjected to SDS-PAGE using a MiniProtein II cell (Biorad, Hercules, CA). The protein bands were visualized using Coomassie blue R-250 staining and were manually excised into fractions according to their apparent molecular weights (up to 116.0 kDa). The polypeptides were then in-gel digested with trypsin and resuspended in 0.1% formic acid (according to Ettan Spot Handling Workstation User Manual 18-1153-55 Edition AC, GE Healthcare Biosciences, Uppsala, Sweden). Nanoscale LC separation of tryptic peptides was performed with a NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C₁₈ 5 μm , 180- $\mu\text{m} \times 20\text{-mm}$ Trap column and a BEH130 C₁₈ 1.7 μm , 100- $\mu\text{m} \times 100\text{-mm}$ analytical reversed phase column (Waters Corp., Milford, MA). In details, the samples were initially transferred to the trap column using mobile phase A (aqueous 0.1% formic acid solution) with a flow rate of 15 $\mu\text{L}/\text{min}$ for 1 min. The peptides were separated with a gradient of 15–50% mobile phase B (acetonitrile with 0.1% formic acid) over 15 min at a flow rate of 600 nL/min followed by a 3 min rinse with 80% of mobile phase B. The column temperature was maintained at 35 °C. All samples of tryptic peptides were analyzed using a SYNAPTTM HDMS mass spectrometer (Waters Corp., Manchester, UK). The system was operated in the V-mode with a resolution of at least 10,000 full-width half-maximum, using positive nanoelectrospray ion mode. The MS/MS survey was over range from 50 to 1990 Da and scan time was 0.5 s. All MS/MS spectra were searched using the Mascot[®] search engine version 2.6 (Matrix Science, Boston, MA) against the Aspergillaceae database in NCBI-nr using the following criteria: enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (± 0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages. The candidate protein queries were mapped to the UniProt Knowledge base (UniProtKB) (Magrane and Consortium 2011). The significance threshold *p* value was <0.05.

Enzymatic hydrolysis of cassava pulp

Hydrolysis reactions (50 mL) were performed in a 250 mL Erlenmeyer flask containing 8–20% (w/v) of cassava pulp in 50 mM sodium acetate buffer, pH 5.0 with a 1× dosage (2.19 mg protein/g substrate on a dried weight basis) of the multi-component enzyme. The reaction mixture was incubated at 40 °C for 96 h with rotary shaking at 200 rpm. Samples were collected at time intervals for reducing sugar and glucose analysis. The total amount of reducing sugar released was determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959).

Viscosity measurement

Effects of the multi-component enzyme on viscosity reduction of cassava pulp were examined under conditions described above at 16% (w/v) solid content in 50 mM sodium acetate buffer, pH 5.0. The substrates were adjusted to pH 5.0 and treated with a varying enzyme dosage from 0.25 to 2.19 mg protein/g substrate, based on a dried weight basis, at 40 °C for 2 h with 200 rpm mixing. Samples were collected after enzyme treatment for viscosity analysis using a commercial stress-controlled rheometer (Bohlin Gemini HR Nano, Malvern Instruments, Malvern, UK). The parameters used for viscosity analysis by the frequency sweep test on the rheometer are as follows: frequency range 0.05–20 rad/s with stress 20 Pa at 25 °C for 15 min using serrated plate geometry with diameter of 25 mm and gap size of 2.0 mm. Reactions with no-enzyme were used as controls (Poonsrisawat et al. 2014). The experiments were done in duplicate. The significant differences of viscosity among samples were tested via one-way ANOVA and post hoc test by Tukey's HSD test.

Scanning electron microscopy analysis

Morphological changes occurring as a result of enzymatic treatment of cassava pulp were observed by scanning electron microscopy. The samples were attached onto stubs and allowed to dry. The specimens were coated with gold to avoid charging under the electron and analyzed on a JSM-6301F scanning electron microscope (JEOL Ltd., Tokyo, Japan) with an acceleration voltage of 20 kV.

Simultaneous saccharification and fermentation (SSF) by a non-thermal process

SSF experiments were carried out in a 1-L bioreactor (Biostat® Q, B.Braun Biotech International) with a working volume of 500 mL. The fermentation mixtures contained 16% (w/v) cassava pulp in 50 mM sodium acetate

buffer, pH 5.0, and 50 mL of basal medium (5 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.025 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 g/L yeast extract). Pre-saccharification of cassava pulp prior to SSF process was performed by adding 2.19 mg protein/g substrate of the multi-component enzyme and incubated at 40 °C for 5 h with an agitation speed of 250 rpm. After pre-saccharification, the temperature was reduced to 30 °C and 50 mL of yeast cell suspension (4.0×10^8 CFU/ml) was added. The fermentation mixture was incubated for 96 h. A control experiment containing 0.25% (w/v) sodium azide as a microbial inhibitor was performed under the same conditions but with no yeast inoculation. Samples were withdrawn at various time intervals (5, 11, 24, 33, 48, 57, 72 and 96 h) for glucose and ethanol analysis. Glucose and ethanol concentration were determined by high performance liquid chromatography equipped with a refractive index detector (Ultimate™ 3000, DIONEX and RI-101, Shodex, Japan) using an Aminex HPX-87H column (Bio-Rad, Hercules, USA) with a Cation H micro-guard cartridge (Bio-Rad, Hercules, USA). The column temperature was maintained at 65°C. Samples were eluted with 5 mM H_2SO_4 in doubly deionized water, which was used as the mobile phase at a flow rate and injection volume of 0.5 mL/min and 20 µL, respectively. A saccharification control containing no yeast inoculum was also performed under the same conditions.

Result and discussion

Biochemical characterization and proteomic analysis of the enzyme

A crude mixture of different polysaccharide hydrolases was obtained by solid-state fermentation using agricultural residues as the major carbon and nitrogen sources with supplements of nutrients and trace minerals. According to our production procedure, 0.65 L of the crude multi-component enzyme with the total protein concentration of 4.48 mg/mL could be extracted from 1 kg of solid-state culture. The enzyme preparation was a complex mixture of starch-degrading and plant cell wall-degrading enzymes containing endo-acting α -amylase, cellulase, pectinase, β -glucanase, and xylanase as the major activities together with various downstream oligosaccharide-processing glycosyl hydrolases which acted cooperatively with the endo-acting enzymes on hydrolysis of starch, cellulose, hemicellulose, and pectin (Table 1).

Proteomic profiling of the fungal secretome done by LC-MS/MS showed that the crude enzyme comprised a complex mixture of hydrolases, lyases, and oxidoreductases targeting on processing of polysaccharides, proteins, and lipid. The majority of them were identified as

Table 1 Activity profiles of crude enzyme produced from *A. aculeatus* BCC17849

Enzyme	Activity (IU/mL)
Fiber degrading enzyme	
Pectinase	224.11 ± 5.58
Cellulase (CMCase)	130.80 ± 3.89
Cellulase (FPase)	0.26 ± 0.02
Xylanase	45.63 ± 3.31
β-Glucanase	463.43 ± 0.80
Mannanase	187.17 ± 5.67
β-Glucosidase	13.49 ± 0.38
β-Xylosidase	0.87 ± 0.05
Starch-degrading enzyme	
Amylase	52.63 ± 1.36
RSD	1.08 ± 0.09

RSD raw starch-degrading amylolytic enzyme

hydrolases with starch- and lignocellulosic polysaccharide-degradation functions. A number of peptides were identified which matched to proteins of *A. niger*, *A. awamori*, and *A. fumigatus* origins in various glycosyl hydrolase (GH) families (Table 2). The amylolytic enzymes identified by proteomics were classified into endo-acting α-amylases (GH13) and exo-acting glucoamylases and α-glucosidases (GH15 and GH31). Some of them have been reported to contain raw starch-binding domains, suggesting their possible function on degradation of raw starch granules (Goto et al. 1994; Juge et al. 2002). A complete cellulolytic enzyme system was annotated in the enzyme extract comprising endo-glucanase (GH5 and GH74), cellobiohydrolase (GH7), and β-glucosidase (GH3). A variety of hemicellulases were also found, including a number of endo- and exo-enzymes attacking heterogeneous hemicelluloses; e.g., endo-β-1,4-mannanase (GH5), β-xylosidase, β-1,4-mannosidase (GH 3), and β-galactosidase (GH 35). In addition, several pectinolytic enzymes were identified, including endo-polygalacturonase (GH28) and rhamnosidase (GH78). The multi-starch and non-starch polysaccharide-degrading composite enzymes in the secretome suggested the ability on efficient cooperative degradation of the fibrous cell wall structure and saccharification of the released raw starch granules from starch-based lignocellulosic substrates such as cassava pulp.

Non-thermal saccharification of cassava pulp

Enzymatic hydrolysis of raw cassava pulp using the multi-component enzyme was studied by varying the initial solid loading of the substrate at a fixed enzyme dosage of 2.19 mg protein/g substrate (equivalent to activities of 26.32 U/g cassava pulp α-amylase, 0.54 U/g RSD activity,

65.40 U/g cellulase, 22.82 U/g hemicellulase, and 112.06 U/g pectinase). Reducing sugars were continuously released during the 96 h reaction, with the highest sugar concentration of 488.8–707.1 mg/g biomass at the end of the reaction when the initial solid was decreased from 20 to 8% (w/v) (Fig. 1). Low reducing sugar yield (<20 mg/g biomass) was obtained under the reaction conditions with no enzyme. Lower conversion yield was obtained at higher solid loading, which could reflect the limitation in mixing in shake-flasks and mass transfer in the reaction due to the water holding capacity of the pulp.

Scanning electron microcopy analysis of the native cassava pulp structure revealed intact spherical raw starch granules encased in the fibrous plant cell wall matrix. Treatment of the pulp by the multi-component enzyme resulted in degradation of the lignocellulosic fibrous structure (noticeable after 24 h treatment), leading to almost complete hydrolysis of starch granules after 96 h of incubation (Fig. 2). The observed changes in substrate microstructure during the treatment suggested that the enzyme partially decomposed the cell wall structure, resulting in the enzyme's higher accessibility to the entrapped starch granules which were then effectively hydrolyzed to fermentable sugars under the experimental condition.

Effects of the multi-enzyme on viscosity

Viscosity of the reaction depends on the solid content and characteristics of the substrate. Efficiency of the multi-component enzyme components on reducing viscosity of the fermentation mixture containing dried cassava pulp at the solid loading of 16% w/v was studied at varying enzyme dosages from 0.125× to 1×, which is equivalent to 0.25–2.19 mg protein/g substrate, for 2 h. Changes in complex viscosity as a function of angular frequency are shown in Fig. 3a. Increasing the multi-component enzyme dosage led to respective decreases in the total solid contents, and hence viscosity of the fermentation mixtures. At 1× and 0.5× enzyme loading, the viscosity was rapidly decreased and the mixture then behaved more liquid-like with less shear thinning. Decrease in enzyme dosage to 0.25 and 0.125× led to respective reduction in viscosity in a measurable range under the experimental conditions.

At a fixed angular frequency of 15.79 rad/s, which is equivalent to the mixing speed of 151 rpm, the maximum reduction of viscosity (99.98%) was achieved by enzymatic treatment with 1× of enzyme loading for 2 h, leading to the decrease in complex viscosity from 3785 Pa s achieved in the control reaction with no enzyme to 0.45 Pa s (Fig. 3b). The result showed that there was statistically significant differences between group means as determined by one-way ANOVA [$F(4,5) = 10.55$, $p = 0.012$]. The

Table 2 Proteomic analysis of *A. aculeatus* BCC17849 secretome by LC–MS/MS

GenBank accession	Search results	Affiliation	Score	Mass	GH
Cellulases and glucanases					
ADX86749	Glucan 1,4-alpha-glucosidase	<i>Aspergillus niger</i>	99	68309	15
AAN16396	Endoglucanase II	<i>Aspergillus aculeatus</i>	58	35981	5
ABG46337	Beta-glucosidase	<i>Aspergillus niger</i>	56	93212	3
A2QH21	Endo-1,3-beta-glucanase eglC	<i>Aspergillus niger</i> CBS 513.88	21	46347	17
ACT68011	Endoglucanase B	<i>Aspergillus niger</i>	44	36559	5
ACT65727	Cellobiohydrolase A	<i>Aspergillus niger</i>	40	48230	7
AAK77227	Endoglucanase C	<i>Aspergillus niger</i>	22	90459	74
GAA85531	1,4-Beta-D-glucan cellobiohydrolase A	<i>Aspergillus kawachii</i> IFO 4308	29	48249	7
AER93028	Beta-glucosidase	<i>Aspergillus aculeatus</i>	66	91274	3
Hemicellulases					
Q2UR38	Exo-1,4-beta-xylosidase xlnD	<i>Aspergillus oryzae</i> RIB40	17	86452	3
CAB46229	Alpha-galactosidase	<i>Aspergillus niger</i>	23	48784	27
ADK88903	Endo-beta-1,4-mannanase	<i>Aspergillus niger</i>	17	41168	5
XP_002375730	Beta-galactosidase	<i>Aspergillus flavus</i> NRRL3357	61	109870	35
XP_001389416	Exo-1,4-beta-xylosidase xlnD	<i>Aspergillus niger</i>	8	87211	3
AAA67426	Mannanase	<i>Aspergillus aculeatus</i>	130	41082	5
Pectinases					
AAG13964	Rhamnosidase B precursor	<i>Aspergillus aculeatus</i>	40	63841	78
O74213	Endopolygalacturonase I	<i>Aspergillus aculeatus</i>	22	38528	28
Starch-degrading enzymes					
AEE60909	Glucoamylase	<i>Aspergillus niger</i>	151	68309	15
BAD06002	Alpha-amylase	<i>Aspergillus awamori</i>	43	54794	13
CBF78451	Glucoamylase (Eurofung)	<i>Aspergillus nidulans</i> FGSC A4	24	70637	15
BAA25205	Glucoamylase	<i>Aspergillus oryzae</i>	23	52402	15
BAD06003	Alpha-amylase	<i>Aspergillus awamori</i>	53	69243	13
AEI58995	Glucoamylase	<i>Aspergillus awamori</i>	23	68315	15
BAM72725	Alpha-glucosidase	<i>Aspergillus niger</i>	43	108913	31

Glycosyl hydrolases related to cell wall degradation are shown

multiple comparison from Tukey's HSD test indicated that the control showed significant different from $0.125\times$, $0.25\times$, $0.5\times$ and $1\times$ (p value <0.05). The target operating viscosity level below 0.5 Pa s, acceptable for efficient mixing in very high gravity fermentation, could also be achieved by a lower enzyme loading ($0.5\times$). The results thus showed the efficiency of the enzyme on reducing viscosity of the fermentation mixture containing high solid loading of cassava pulp simultaneously with the hydrolysis and saccharification of the starch and cellulosic fractions.

Simultaneous saccharification and fermentation of raw cassava pulp

The applicability of the multi-component enzyme in ethanol fermentation was studied using the non-thermal (uncooked) SSF process (Rattanachomsri et al. 2009). In these

experiments, a laboratory-scale bioreactor at an initial solid loading of 16% (w/v) was used with no prior high-temperature pre-gelatinization/liquefaction steps. Starch was present mainly in the insoluble and non-gelatinized form during the process. A higher saccharification efficiency of the substrate was achieved in the bioreactor compared with the small-scale experiment, probably owing to improved mixing efficiency with more shearing in the bioreactor. Under these conditions, the maximum total sugar concentration of 892.7 mg/g biomass was obtained in a control experiment in the presence of sodium azide as a microbial inhibitor with no yeast inoculation. Glucose was the major fermentable sugar product with the final concentration of 736.4 mg/g biomass and is equivalent to the conversion yield of 82.92% from starch and cellulose. This indicated that the starch fraction was almost completely saccharified with partial hydrolysis of the non-starch glucan fraction.

Fig. 1 Reducing sugars released from enzymatic hydrolysis using *A. aculeatus* multi-enzyme components. The reactions contained 2.19 mg protein of the multi-component enzyme/g substrate on a dried weight basis with 8–20% (w/v) cassava pulp in 50 mM sodium acetate buffer, pH 5.0 and incubated at 40 °C for 96 h with rotary shaking at 200 rpm

Xylose and arabinose from hydrolysis of the hemicellulose were obtained as minor sugar products from saccharification of the pulp.

In the non-thermal SSF process, glucose released from the cassava pulp by the enzyme was simultaneously converted to ethanol by *S. cerevisiae*. A rapid increase in ethanol concentration was observed during the first 48 h fermentation period with concomitant consumption of glucose (Fig. 4). A residual glucose level of 0.079 mg/g biomass was found at the end of fermentation. Nevertheless, a high ethanol concentration of 6.98% w/v in the final hydrolysate was achieved. This was equivalent to 0.44 g/g substrate or 96.7% of the theoretical yield based on the total starch and cellulose content with the ethanol productivity of 0.727 g/L/h. Overall, the results demonstrated the potential of using the multi-component enzyme for bioethanol production from cassava pulp by the non-thermal processes at initial high solid loading.

Compared to fresh roots and chips, the pulp contains higher lignocellulosic content with entrapped starch inside the fibrous plant cell wall structure. Processing pulp presented more difficulties in pumping and agitation due to its high solid content and high water binding capacity. The pulp is also more resistant to enzymatic degradation owing to its high plant cell wall polysaccharide content. Due to its structural and physicochemical characteristics, cassava starch granules are also less susceptible to enzymatic hydrolysis compared to cereal starches (Piyachomkwan et al. 2007) due to an absence of peripheral pores and channels which enable α -amylase penetration (Lehmann and Robin 2007). The use of *A. aculeatus* enzyme on non-

Fig. 2 Scanning microscopy analysis of cassava pulp structure before (a) and after non-thermal saccharification by *A. aculeatus* multi-component enzyme for 24 h (b) and 96 h (c)

thermal saccharification of cassava pulp was first reported in small-scale experiments which showed its high capability on releasing fermentable sugars compared to combinations of various commercial starch and lignocellulose degrading enzymes (Rattanachomsri et al. 2009). Application of the multi-component enzyme on reducing viscosity of fresh cassava pulp as well as fresh root mash and chips in a very high gravity fermentation process has been recently reported (Poonsrisawat et al. 2014). A high viscosity in reactions containing lignocellulosic materials is related to the high water holding characteristic and high gelling capacity of the biomass. Various cell wall-degrading activities including cellulase, pectinase (polygalacturonase), xylanase, cellobiohydrolase, and mannanase were shown to degrade cell wall fibers of cassava pulp (Divya Nair et al. 2011) and other root mashes (Gilbert 2010; Cao et al. 2011) composed of a complex lignocellulosic matrix with high pectin content (30–50%). Treatment of cassava pulp with cellulase and pectinase was shown to disrupt the

Fig. 3 Effects of the *A. aculeatus* multi-component enzyme on viscosity reduction. **a** Complex viscosity profile of 16% w/v dried cassava pulp with varying enzyme dosages (0.25–2.19 mg protein of the multi-component enzyme/g substrate) at pH 5.0, 40 °C for 2 h. The average data from duplicate independent experiments are shown; **b** comparison on complex viscosity reduction of 16% (w/v) dried cassava pulp at 15.79 rad/s pretreated with different enzyme dosages. The average data from duplicate independent experiments are shown. Groups with the same letters above bars indicate no significant difference ($p < 0.05$) according to Turkey's test

cell wall matrix, resulting in the release of starch granules from the substrate and increased α -amylase hydrolysis products (Sriroth et al. 2000b). Combination of commercial cellulase and raw starch-degrading amylase was also reported for co-digestion of the cellulose and raw starch in cassava pulp (Divya Nair et al. 2012). These works thus suggests the efficient cooperative action of a variety of endo- and exo-acting cell wall-degrading and raw starch-hydrolyzing enzymes in the fungal enzyme preparation as shown by our proteomic analysis on hydrolysis of this unique starch-cellulosic substrate.

The SSF strategy has been shown to improve yeast performance and alleviate feedback inhibition of the hydrolytic enzymes by highly accumulated sugar level with advantages on improved productivity compared with the conventional separated hydrolysis and fermentation process (Lin and Tanaka 2006). Coupling the direct non-thermal saccharification process to SSF fermentation can reduce energy input overall by 20% in the case of using

Fig. 4 Non-thermal simultaneous saccharification and fermentation of cassava pulp. Cassava pulp (16% w/v) was pre-saccharified with 2.19 mg protein of the multi-component enzyme/g substrate on a dried weight basis and incubated at 40 °C, pH 5.0 for 5 h with agitation at 250 rpm. The pretreated mash was used as the substrate for ethanol fermentation using *S. cerevisiae* in a 1-L bioreactor with a total volume of 500 mL at 30 °C for 96 h

cassava root mash as the raw material due to the absence of pre-gelatinization/liquefaction steps, resulting in considerable energy saving and improved economic feasibility (Poonsrisawat et al. 2014). Ethanol fermentation from cassava pulp by a non-thermal SSF process was first reported using the enzyme prepared by submerged fermentation of *A. aculeatus* with *Candida tropicalis* as an ethanogen; however, the initial pulp loading was low (Rattanachomsri et al. 2009). Ethanol production from raw cassava flour (18.2% dried solid) by the SSF process using a complex enzyme from *Aspergillus kawachii* consisting of glucoamylase, acid-stable α -amylase, cellulase, xylanase, and protease activities in pilot scale was also reported with high efficiency (Sugimoto et al. 2012). The ethanol yield obtained from our study was relatively high compared with other reports of ethanolic fermentation of raw starches from cassava and other monocotyledonous or dicotyledonous plants e.g. corn and sweet potato using the uncooked non-thermal process with α -amylases and glucoamylases from other microbes e.g. *Streptococcus bovis*, *R. oryzae*, and *A. awamori* (Shigechi et al. 2004; Khaw et al. 2007; He et al. 2009) or recombinant ethanologens expressing heterologous raw starch-hydrolyzing amylases from *A. niger*, *Debaryomyces occidentalis*, and *A. tubingenensis* which were in the range of 6.7–66.5 g/L, corresponding to the final ethanol concentration of 0.8–6.2% (Viktor et al. 2013; Wang et al. 2014). An ethanol concentration of 12.9 g/L was reported for ethanol fermentation from cassava pulp combined with cassava waste water using one-step enzyme hydrolysis process with a high-

temperature pre-gelatinization/liquefaction step (Virunanon et al. 2013). The slower rate in starch hydrolysis in the non-thermal process incurs a delay of fermentation, and the increased ethanol stress during the longer fermentation period negatively impacts on yeast physiology.

The efficiency of the non-thermal SSF process can be further improved by several approaches including optimization of media and fermentation conditions for increasing enzyme productivity and searching for enzymes with higher efficiency and specificity on degradation of cassava starch granules for the formulation of a more efficient enzyme system, which can result in reduction of the enzyme cost. The developed process can be further modified by improving process design for more efficient mixing and selecting yeast strains with higher tolerance to ethanol toxicity to increase the process efficiency for ethanol production.

Conclusion

A complex cell wall degrading and raw starch-hydrolyzing enzyme from *A. aculeatus* was applied for efficient ethanol production from cassava pulp at a high initial solid loading using the alternative, non-thermal SSF process with simultaneous viscosity reduction. This, in overall, could effectively result in a less energy intensive process. The simple and efficient enzymatic method developed in this study provides a promising strategy for the conversion of cassava pulp to various fermentation products with improved process efficiency and economics.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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