Enzyme Matching Design Approach on Very High Gravity Liquefaction and Saccharification of Cassava Root and Cassava Starch for Ethanol Fermentation

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During ethanol production, the ratio of various sources of starch-hydrolyzing enzymes significantly influences hydrolysis efficiency in a very high gravity (VHG) system. In this study, the enzyme matching approach was proposed to optimize liquefaction and saccharification yield of cassava root and cassava starch. For fresh cassava root, the synergistic effect of using Techzyme Q-Add enzyme (93 °C pH 5.6) for liquefaction and GC 147 enzyme (61.5 °C pH 4.2) for saccharification achieved the highest yield among all enzyme-matching designs. At 25% solid, highest total reducing sugar (TRS) yields from sequential liquefaction and saccharification of cassava starch, fresh cassava, and dry cassava were 87.9%, 85.1%, and 70.0% corresponding to 286.8, 249.4, and 241.1 g/L, respectively. For VHG ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5606, separate liquefaction and simultaneous saccharification and fermentation (SLSSF) gave a significantly higher ethanol concentration compared with separate liquefaction and saccharification and fermentation (SLSF). From a 35% solid SLSSF system, the highest ethanol produced at 30 °C was 27.3 g/L from 72 h, respectively. The results suggested that SLSSF could effectively shorten the time course of the whole process for liquefaction, saccharification, and fermentation from 74 h to only 26 h for similar ethanol production yields.

Keywords: Cassava root and cassava starch; Enzyme matching design approach; Ethanol fermentation; Separate and Simultaneous liquefaction; Saccharification and fermentation; High gravity

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INTRODUCTION

Cassava (*Manihot esculenta* Crantz) has been cultivated worldwide in subtropical and tropical regions as food and feedstock. It has been ranked fourth among all global cultivar production (Whankaew *et al.* 2011). Due to climate change, the global agricultural productivity has been significantly influenced by drought. Cassava is one of the most drought tolerant cultivars that can be planted in most areas having insufficient water supply. Apart from its availability, its easily hydrolysable characteristics makes cassava an ideal substrate for biochemical conversion to various products as well as biofuels, *e.g.*, ethanol and butanol. Traditionally, fresh cassava is widely utilized in the food industry by the starch extraction process, while dry cassava chip is mostly used as feedstock for fermentation, which requires long-term storage. Utilization of fresh and dry cassava root instead of cassava starch as substrate for microbial processes to produce chemical commodities with new technology and process optimization approach is considered a crucial aspect to produce ethanol as renewable fuel. However, the extraction of starch from tuber crops is a problem due to the latex and trapping of starch granules in the pectin-hemicellulose

matrix. The use of mixed enzyme complexes enhances starch hydrolysis yield and reduces resistant starch (Poonsrisawat *et al.* 2014).

The technological and bioprocess design approaches have been used to enhance ethanol production efficiency in terms of low cost, near-zero waste process, and short reaction. VHG liquefaction and saccharification along with fermentation have been recently proposed for most starchy biomass utilization, *e.g.*, potato (Srichuwong *et al.* 2009; Watanabe *et al.* 2010), corn (Kollaras *et al.* 2011), wheat (Jones and Ingledew 1994; Pietrzak and Kawa-Rygielska 2015), sorghum, barley, *etc*. This system increases ethanol concentration in the mash without needing to scale up the fermenter (Bao *et al.* 2011). Moreover, it requires lower energy input for downstream ethanol distillation. The limitation of utilizing VHG technology is incomplete fermentation regarding several stresses toward yeast cells. For example, osmotic stress due to very high concentrations of sugar or dissolved solids causes increasing external osmotic pressure. Other reasons are loss of cell viability, slow growth rate, or inadequate performance of yeast due to the toxic level of ethanol in the fermentation broth. Consequently, there are high concentrations of residual sugar at the end of fermentation, which is called stuck fermentation (Gibson *et al.* 2007). Few studies have been conducted on VHG fermentation of cassava root and cassava starch (Nguyen *et al.* 2014; Poonsrisawat *et al.* 2014).

To produce fuel-grade ethanol, a variety of sources of commercial amylase and glucoamylase from various microorganisms with different optimal pH and temperatures are used. Some examples are Spezyme ® Fred (an α-amylase), Optidex® L-400 (glucoamylase) (Zabed *et al*. 2016), Spezyme® Xtra (a highly powerful α-amylase), Stargen™ 001 (Granular Starch Hydrolyzing Enzyme) (Gennencor) (Shanavas *et al*. 2011), Spezyme® Alpha (Dupont), Liquozyme SC (Novozyme), Spirizyme Fuel (Novozyme), and Pectinex Ultra SP-L (Novozyme) (Srichuwong *et al.* 2009). Different starch hydrolyzing enzymes from diverse sources contain different amounts and types of enzyme cocktails, which substantially affect starch hydrolysis efficiency, especially for VHG liquefaction and saccharification in which viscosity and water assimilation play a key role on the reaction competence. In addition to α-amylase and glucosidase, the enzyme complexes needed to enhance hydrolysis of tuber crops are β-glucosidase, endoglucanase, exoglucanase, xylanases, and pectinases (Poonsrisawat *et al.* 2014; 2016).

During VHG liquefaction and saccharification for ethanol production, insufficient small units of saccharide contents, especially glucose, maltose, and maltotriose, prolong fermentation time or suppress ethanol production of yeast, which leads to low ethanol yield. However, concentrations that are too high in mono-, di-, and tri-saccharides caused increased osmotic stress on yeast (Hounsa *et al.* 1998) and subsequently substrate inhibition (Thatipamala *et al.* 1992). In addition, high concentration of oligosaccharides, as mentioned before, could also re-polymerize onto resistant starch in mash and lead to hydrolysis inhibition (Sharma *et al.* 2010). Several bioprocess techniques have been proposed to solve the osmotic stress problems such as application of physical and chemical factors, *i.e.*, proteins (nitrogen source), polysaccharides (Hounsa *et al.* 1998), fatty acids, or polyphenol compounds (Reddy and Reddy 2006); continuous flow immobilized cell bioreactor (Meethit *et al.* 2016); or two-stage fermentation in one bioreactor (*i.e.*, sequential culture) (Fu and Peiris 2007). To optimize the fermentation efficiency of very high gravity mash, the simultaneous saccharification and fermentation (SSF) platform has been widely used to maintain appropriate balance between oligosaccharides formation from saccharification and their consumption for ethanol production.

In the present study, the enzyme matching design approach was applied to achieve high reducing sugar yield of the VHG liquefaction and saccharification of fresh cassava root, dry cassava root, and cassava starch. To achieve cost effective and high productivity ethanol

production processing from starchy biomass, this study also investigated the efficiency of two different systems: 1) separate liquefaction and simultaneous saccharification and fermentation (SLSSF), and 2) separate liquefaction and saccharification and fermentation (SLSF).

EXPERIMENTAL

Materials

A cassava (*Manihot esculenta* Crantz) Hanatee variety with an 18-month harvesting time was planted in a demonstration field in Rayong Province, Thailand. Three types of alpha-amylase enzymes were used: Techzyme Q-Add (Siam Victory Chemicals Co., Ltd., Bangkok, Thailand), Alpha-Extra enzyme (Siam Victory Chemicals Co., Ltd., Bangkok, Thailand), and Spezyme® Alpha (DuPont, Delaware, USA). These enzymes contain a thermostable starch hydrolyzing α-amylase that is produced by a genetically modified strain of *Bacillus licheniformis* with an activity of > 150,000 AAU/mL (Techzyme Q-Add and Alpha-Extra) or > 13,775 AAU/g (Spezyme® Alpha). For starch saccharification, two types of enzymes were studied: (1) GC 147 (Genencor, New York, USA), which is a blend of enzymes produced by controlled fermentation of genetically modified strains of *Bacillus licheniformis* and *Trichoderma reesei* with activity 580 TGAU/g; and (2) GA extra (Siam Victory Chemicals Co., Ltd., Bangkok, Thailand), which is a blend of enzymes produced by controlled fermentation of genetically modified strains of *Bacillus licheniformis* with activity > 170,000 U/mL. *Saccharomyces cerevisiae* (TISTR 5606) used as inoculum for ethanol fermentation was supplied from the Thailand Institute of Science and Technological Research (TISTR, Pathumthani, Thailand).

Sodium acetate, acetic acid (glacial), dinitrosalicylic acid, maltose, xylose, yeast extract, and peptone were purchased from Merck (Darmstadt, Germany). Phenol, sodium sulfite, potassium dihydrogen phosphate, and sodium hydroxide were purchased from Alpha (Massachusetts, USA). Sodium potassium tartrate, urea, hydrochloric acid, sodium lauryl sulphate, and cetyl trimethylammonium bromide were purchased from Ajax Finechem (Taren Point, Australia). Acetone, sulfuric acid, ethanol, and butanol (GC standard) were purchased from RCI Labscan (Bangkok, Thailand). Dextrins, disodium ethylene tetraacetate (EDTA), and disodium phosphate were purchased from Daejung (Gyeonggi-do, Korea). Maltotriose, arabinose, 2-Ethoxyethanol, and sodium borate decahydrate were purchased from Alfa Aesar (Beijing, China). Glucose was purchased from Sigma Aldrich (Singapore). Octanol was purchased from Applichem (Darmstadt, Germany).

Cassava Mash Preparation from Fresh and Dry Roots

Fresh cassava root was chopped, ground, and stored in a freezer at -20 °C. To prepare the dry cassava root, fresh cassava root after grinding was sundried to approximately 15 to 20% moisture content, processed using a centrifugal mill (Retsch GmbH 5657-HAAN, Haan, Germany), and sieved to -70 / +230 mesh. For long-term storage of substrate, dry cassava was oven-dried at 50 °C for 3 h to 9.4 to 10.6% moisture content. Dry cassava root was stored in a digital desiccator chamber at 40% relative humidity. Cassava starch was purchased from Choothin Co., Ltd., Bangkok, Thailand.

Enzyme Characterization

The molecular weight of α-amylase and glucoamylase used in the experiments was determined with electrophoretic mobility at 200 V for 50 min. Standard proteins (Genedirex)

having known molecular weights were used for comparison. SDS-PAGE was carried out for 10 μg protein/lane using NuPAGE 4 to 12% Bis-Tris gel (Invitrogen, Waltham, MA, USA). Enzyme activity was assayed by measuring the reducing sugar released during cassava starch hydrolysis. The assay mixture consisted of 2%(w/v) cassava starch in 0.1 M acetate buffer pH 5.5 and 0.1 μL of enzyme. The reaction took place at 55 °C for 30 min and suddenly the reaction was stopped by adding 3 mL of DNS reagent and boiling for 5 min (Miller 1959). A colorimetric assay was conducted to measure the reducing sugar released into the supernatant using glucose as standard. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 mmol reducing sugar (glucose equivalence) per min under the standard assay conditions (Marlida *et al*. 2000). The protein content in each amylase and glucoamylase enzyme was measured according to the Bradford method by addition an exact volume of enzyme to react with Bradford reagent. Amount of protein content was calculated from the absorbant at 595 nm when bovine serum albumin was used as the standard (Kruger 1994).

Liquefaction and Saccharification of Cassava Mash and Cassava Starch

Fresh or dry cassava root and cassava starch were liquefied using different commercial amylase enzymes (Techzyme Q-Add, Alpha Extra, and Spezyme® Alpha) and subsequently saccharified using different commercial glucoamylase enzymes (GC 147 and GA extra) with various solid contents (25, 30, and 35%). The enzyme matching design approach was used to optimize the yield of liquefaction and saccharification, as shown in Table 1. Total reducing sugar (TRS) concentration was measured by DNS method, and then the TRS yield was calculated based on the initial concentration of substrate as shown in Eq. 1. The optimal hydrolysis conditions for each enzyme were provided by the manufacturer.

Table 1. Enzyme Matching Design for Optimization of Liquefaction and Saccharification of Cassava Root and Cassava Starch Containing 25, 30, and 35% Solid Content

Saccharification (48 h)	Liquefaction (1 and 2 h)		
	Techzyme Q-Add (93 °C, pH 5.6)	Alpha Extra (93 °C pH, 5.6)	Spezyme® Alpha (84 °C, pH 5.7)
GC 147 (61.5 °C, pH 4.2)	✓	✓	✓
GA extra (52.5 °C, pH 4.7)	✓	✓	✓

$$TRS\ yield\ (\%) = \frac{TRS\ concentration\ (gL^{-1})}{Initial\ substrate\ concentration\ (gL^{-1})} \times 100 \quad (1)$$

The first experiment used fresh cassava root as substrate and varying solid content at 25, 30, and 35% with the enzyme matching design approach (Table 1). After that, a comparison study was performed using fresh cassava root, dry cassava root, or cassava starch as the substrate. The most suitable solid content was selected from the first set of experiments. All the treatments were conducted with two replicates. A schematic diagram of overall experiments is given in Fig. 1.

Batch Ethanol Fermentation by SLSF and SLSSF System

For inoculum preparation, 1 mL of inoculum stored in a 10% glycerol solution was transferred to a 4 mL sterile YPD medium containing 5 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose.

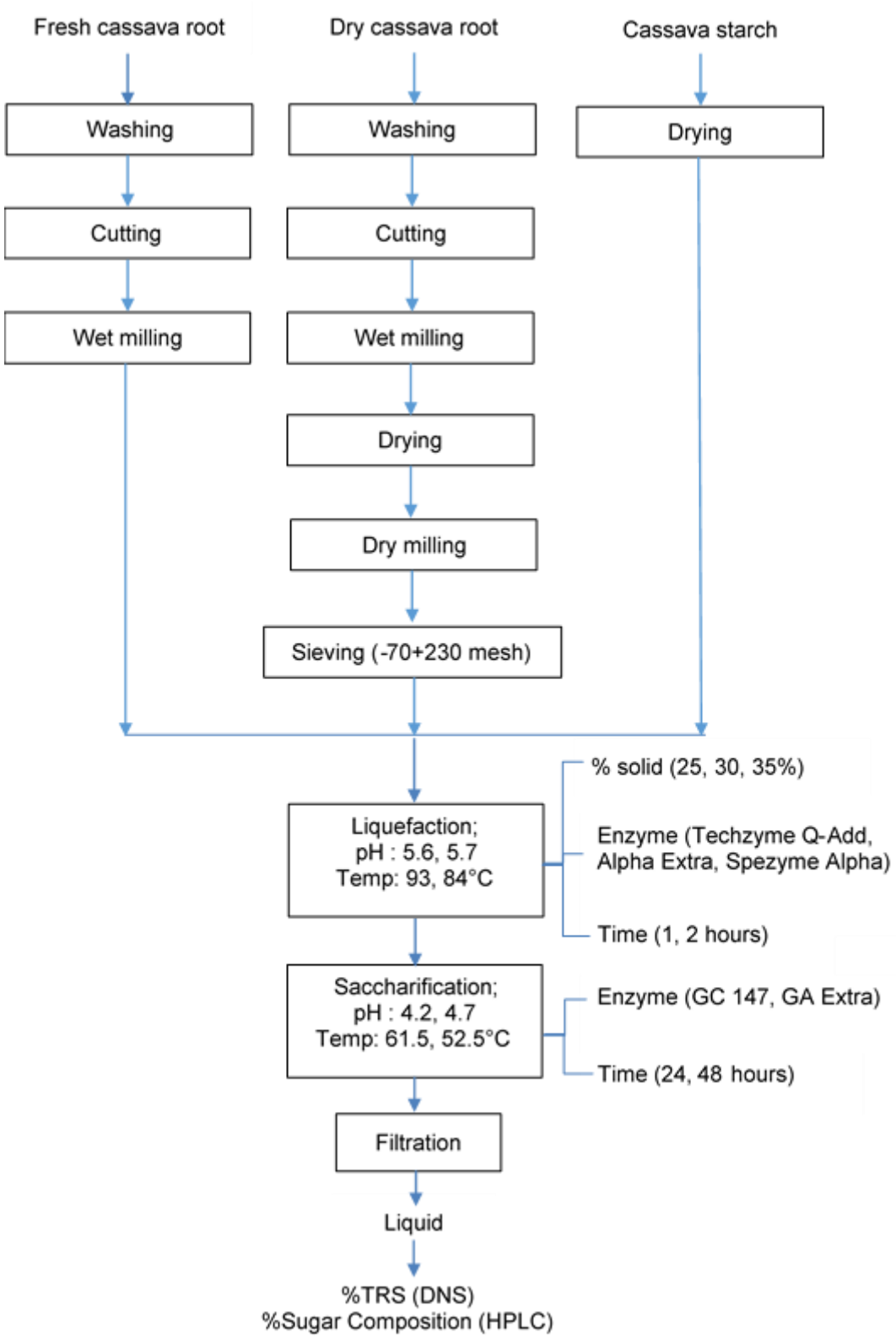

Fig. 1. Liquefaction and saccharification of fresh cassava root, dry cassava root, and cassava starch using enzyme matching design approach

The cultivation took place at 37 °C for 48 h in static conditions. Next, 5 mL of inoculum was transferred to 45 mL of medium and incubated at 37 °C and 100 rpm for 24 h. A total of 10 mL of this inoculum was transferred to 90 mL of medium and again incubated at 37 °C and 100 rpm for 24 h. The prepared stock solution was ready to use as inoculum seed for ethanol fermentation.

Batch ethanol fermentation was performed in a 125 mL Erlenmeyer flask with an air lock stopper. In the first set of experiments using separate liquefaction and saccharification and fermentation (SLSF), dry cassava root was liquefied using Techzyme Q-Add enzyme at pH 5.6, 93 °C for 2 h and then saccharified using GC 147 enzyme at pH 4.2, 61.5 °C for 48 h. Subsequently, 100 mL of saccharified dry cassava root solution was sterilized and used as substrate for ethanol fermentation. The second set of experiments was separate liquefaction and simultaneous saccharification and fermentation (SLSSF), in which dry cassava root was first liquefied by Techzyme Q-Add enzyme at pH 5.6, 93 °C for 2 h. The liquefaction solution was sterilized and used as a medium for simultaneous saccharification and ethanol fermentation at 37°C. All treatments were conducted with two replicates.

Inoculation was performed by adding 10 mL of stock culture into 90 mL of starch suspension prepared by the two methods described above. Samples were taken every 24 h until 96 h of fermentation. Cell counts and pH measurements were performed during the fermentation period. For ethanol fermentation, all treatments were conducted with two replicates. To separate liquid and solid residue, fermented dry cassava root was centrifuged at 8,000 rpm under a controlled temperature of 4 °C, and the supernatant was filtered with a 0.2 μm nylon membrane prior to HPLC analysis. The supernatant was stored at -20 °C for the analysis of sugar and ethanol concentration along with other byproducts. The overall experimental design for batch ethanol fermentation is shown in Fig. 2.

Analytical Methods

Amylose content of cassava root and cassava starch was determined according to the procedure described by McGrance *et al.* (1998). Fiber, lignin, and ash contents were analyzed by the Goering and Van Soest method (Goering and Van Soest 1970). Starch contents followed the standard AOAC method (AOAC 2000). Fat and protein contents were analyzed by acid hydrolysis and solvent extraction by using the Soxtec™ and Kjeldahl methods, respectively. The viscosity of the starch suspension was measured by Brookfield viscometer (RVDV-II+, Massachusetts, USA). Total reducing sugar (TRS) content expressed as glucose equivalence after liquefaction and saccharification of cassava root and cassava starch was analyzed by the dinitrosalicylic acid (DNS) method (Miller 1959). Glucose, maltose, maltotriose, dextrins, acetic acid, and ethanol concentrations during batch ethanol fermentation were analyzed by high performance liquid chromatography (HPLC, Waters e2695, Massachusetts, USA) using an Aminex HPX-87H column 300 mm × 7.8 mm (ID) (BioRad, Hercules, CA, USA) at column temperatures of 60 °C with 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. A reflective index (RI) detector (Waters 2414) was used at a detector temperature of 40 °C. The ethanol yield ($Y_{p/s}$) was calculated from ethanol produced (g) based on sugars consumed (g) as shown in Eq. 2,

$$Y_{P/S} = \frac{P_f - P_i}{S_r - S_i} \quad (2)$$

where P_i and P_f are initial and final ethanol concentrations (g/L), respectively. S_i and S_r are initial and final sugar concentrations (g/L), respectively.

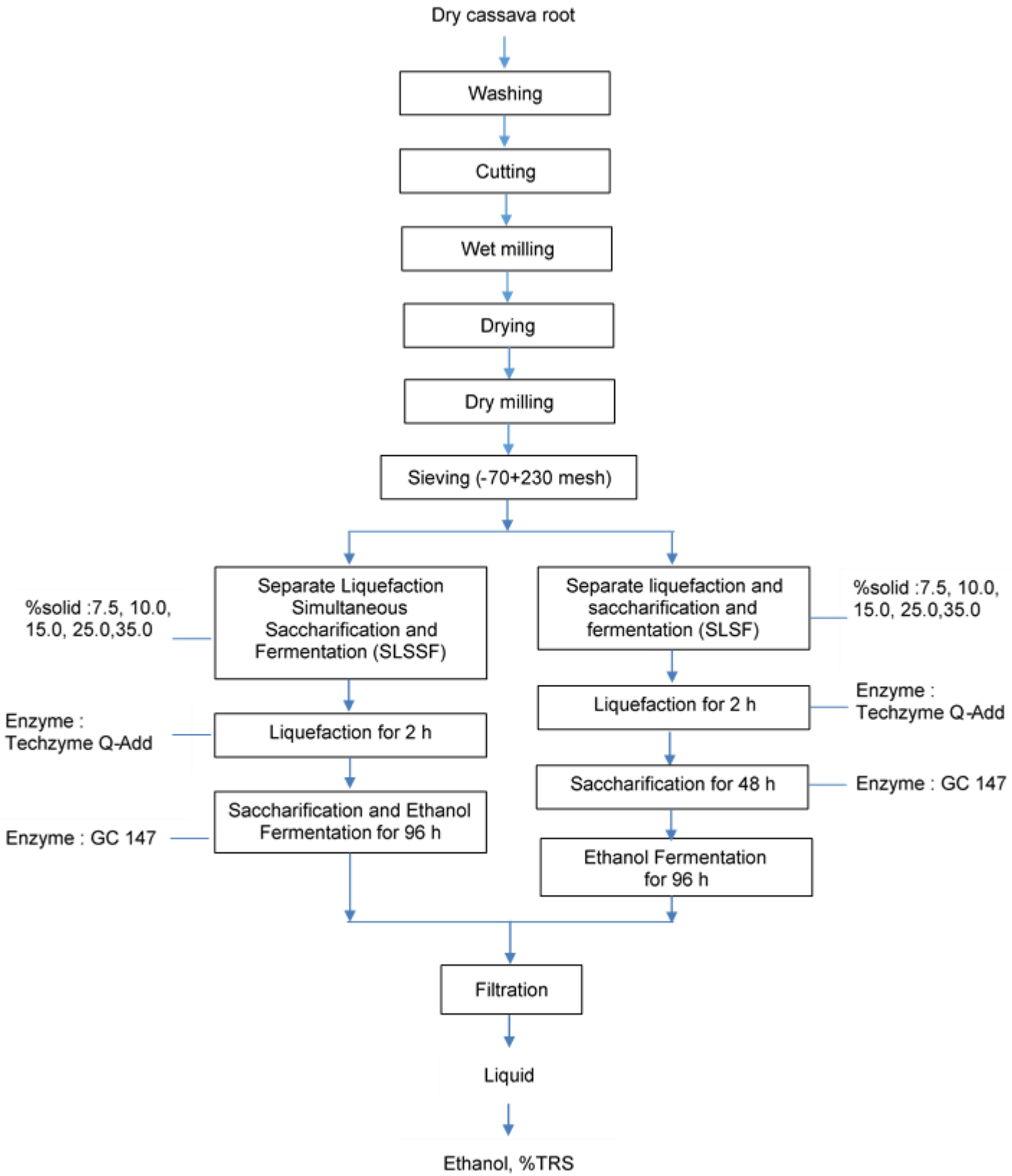

Fig. 2. SLSF and SLSSF system for batch ethanol fermentation

Scanning electron microscopic (SEM) analysis was applied to analyze starch granular changes during liquefaction and saccharification of cassava. The sample was dried gently at 50 °C for 2 to 3 days before analysis. Gold coating was performed for at least a 10 nm thick layer using a 15 min duration prior to image analysis with a scanning electron microscope at 12.5 kV (FEI, Quanta 450, Hillsboro, OR, USA).

Statistical and Computational Analysis

All values presented in all tables and figures are average values and standard deviation (S.D.) and were computed based on two replicates. Analysis of variance (ANOVA) was performed according to the Tukey test at a significant level of 0.05 (PASW Statistics 18.0, SPSS Inc., Chicago, USA) to evaluate the significance of differences of average values in each group. A heat map of TRS yield from enzyme matching approach for liquefaction and saccharification was generated by programming in MATLAB 2017 (Natick, MA, USA) to analyze the most appropriate matching.

RESULTS AND DISCUSSION

Chemical Composition of Cassava Root and Cassava Starch

The chemical composition of cassava root and cassava starch is shown in Table 2. The starch content of cassava root and cassava starch were 81.3% and 87.6%, respectively. Some amount of cellulose (2.13%), hemicellulose (0.83%), and lignin (0.71%) were found in cassava root as it included cassava peel and fiber. Higher amounts of ash were observed for cassava root over cassava starch. The results obtained are in good agreement with a previous work which reported that protein, lipid, ash, crude fiber, and starch of Hanatee cassava flour at 12-month harvest time were 0.94 ± 0.00, 0.34 ± 0.01, 2.02 ± 0.01, 2.18 ± 0.08, and 82.41 ± 0.42 g/100g dry weight (Charoenkul *et al*. 2011). Therefore, in cassava root liquefaction and saccharification, cellulosic enzyme may have need for the better release of starch granule that facilitates higher yield of liquefaction and saccharification, apart from amylolytic enzyme.

Enzyme Characterization

From the analysis, the activity of commercial amylase namely Spezyme® Alpha, Alpha-Extra, and Techzyme Q-Add were 11700, 8340, and 15700 μL^{-1}, respectively. Activities of commercial glucoamylase, namely GC Extra and GC 147, were 10160 and 11890 μL^{-1}. The protein concentrations of amylase, namely Spezyme® Alpha, Alpha-Extra, and Techzyme Q-Add, were 2540, 2410, and 2510 μg/mL, respectively, while GC Extra and GC 147 (glucoamylase) contained 3070 and 3300 μg/mL, respectively. Therefore, Spezyme® Alpha, Alpha-Extra, and Techzyme Q-Add contained 4610, 3460, and 6260 U g^{-1} protein, respectively. For glucoamylase, GC Extra and GC 147 contained 3310 and 3601 U g^{-1} protein, respectively.

From electrophoretic analysis, all amylase enzymes used in the study, including Spezyme® Alpha (Lane C3), Alpha-Extra (Lane C4), and Techzyme Q-Add (Lane C5), contain extracellular α-amylase, which was determined as 56,000 Da compared to the protein marker as shown in Fig. 3. The results are in good accordance with molecular weight of extracellular α-amylase isolated from *Bacillus subtilis* KIBGE HAS (Bano *et al*. 2011). The molecular weight of extracellular α-amylase from all commercial amylase enzyme investigated was quite close to the molecular weight of α-amylase (55,000 Da and 57,000 Da) isolated from *B. subtilis* PKTH 10 and *Streptomyces gulbargensis*, respectively from the previous studies (Takkinen *et al.* 1983; Syed *et al*. 2009).

Table 2. Chemical Composition of Cassava Root and Cassava Starch

Components	Content (% dry basis)	
	Cassava Root	Cassava Starch
Starch	81.26 ± 4.06	87.60 ± 8.62
- Amylose	18.03	25.24
- Amylopectin	34.23	32.36
Protein	0.81	0.10
Fat	ND	ND
Ash	4.61 ± 0.17	0.21 ± 0.00
Natural Detergent Fiber (NDF)	4.30 ± 0.09	ND
Acid Detergent Fiber (ADF)	3.46 ± 0.03	ND
Lignin	0.71 ± 0.02	ND
Cellulose	2.13 ± 0.03	ND
Hemicellulose	0.83 ± 0.07	ND
Moisture	9.15	9.96

ND = Not Detected

The molecular weights of the glucoamylase enzymes GA extra (Lane C1) and GC 147 (Lane C2) were similar at 40,000 and 47,000 Da (Fig. 3). The findings are in good agreement with a previous study stating that purified glucoamylase from *Lactobacillus amylovorus* was a single protein band of molecular weight 47,000 Da (James *et al*. 1997). Another report showed that protein having molecular weights of 42,800 and 42,000 Da isolated from *B. subtilis* DM-03 and *Bacillus* sp. TS-23 showed amylolytic enzyme activity and could possibly be glucoamylase enzymes (Krowczynska *et al*. 1995; Laemmli 1970).

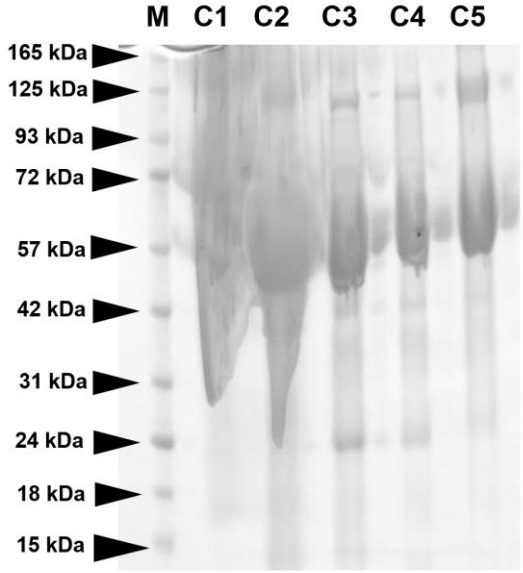

Fig. 3. Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns of different amylase and glucoamylase enzymes, on 4 to 12% Bio-Tris gel with SimplyBlue™ SafeStain. Lanes: M, marker proteins; C1, GA extra glucoamylase; C2, GC 147 glucoamylase; C3, Spezyme® Alpha amylase; C4, Alpha-Extra amylase; and C5, Techzyme Q-Add amylase.

The ability to dextrinise and saccharify starch was also found for protein activities in the 71-kDa glucoamylase and a 53-kDa alpha-amylase-like enzyme supported by antibody cross-reactivity studies (Dubey *et al*. 2000). Moreover, a 125-kDa starch hydrolysing enzyme produced by *Aspergillus niger* and characterised by its ability to dextrinise and saccharify starch was also found to possess activity towards raw starch (Suresh *et al*. 1999). This kind of enzyme was found in significant amounts in all amylase enzymes tested in the present study, namely Spezyme® Alpha (Lane C3), Alpha-Extra (Lane C4), and Techzyme Q-Add (Lane C5).

VHG Liquefaction and Saccharification of Fresh Cassava Root

The enzyme matching design approach was applied to optimize TRS yield from sequential liquefaction and saccharification of fresh cassava root containing different solid contents (25, 30, and 35% total solid). Table 3 shows TRS yields from fresh cassava root hydrolysis by Spezyme® Alpha, Alpha Extra, and Techzyme Q-Add liquefaction (1 h and 2 h) and subsequent 48 h saccharification by GC 147 and GA Extra at 25%, 30%, and 35% solid. Table 3 shows that TRS yield increased rapidly in the first hour of liquefaction, and TRS yield was slightly increased and kept steady at 2 h liquefaction. However, the highest TRS yield was obtained at 2 h liquefaction with Techzyme Q-Add. The highest TRS yield was increased 1.5 to 2.0 times at 24 h saccharification by GC 147, and the TRS yield was slightly increased 0.1 to 15.7% at 48 h saccharification (Fig. S1). The highest TRS was 85.13% ± 0.98 w/w cassava dry basis at 25% solid. The highest TRS concentration in fresh cassava root, dry cassava root, and cassava starch hydrolysate at 35% solid were 249.4 ± 0.2, 241.1 ± 1.0, and 286.8 ± 11.3 g/L, respectively.

Table 3. TRS Yields from Fresh Cassava Root Hydrolysis by Spezyme® Alpha, Alpha Extra, and Techzyme Q-Add Liquefaction (1 h and 2 h) and Subsequent 48 h Saccharification by GC 147 and GA Extra

		Total reducing sugar (TRS) yields (%w/w)					
α-Amylase		Techzyme Q-Add [a]		Alpha Extra [b]		Spezyme® Alpha [c]	
Gluco-amylase	Liquefaction Time (h)	1 h	2 h	1 h	2 h	1 h	2 h
GC 147 [d]	25% Solid	77.0% ± 1.4	85.1% ± 1.0	62.4% ± 5.4	62.9% ± 0.2	55.7% ± 0.2	57.6% ± 2.4
	30% Solid	72.7% ± 2.8	78.7% ± 1.0	64.0% ± 2.6	59.2% ± 0.7	49.0% ± 6.8	55.6% ± 2.3
	35% Solid	69.7% ± 3.9	71.0% ± 0.5	60.1% ± 0.5	56.9% ± 2.2	50.4% ± 2.1	52.4% ± 1.4
GA Extra [e]	25% Solid	76.6% ± 3.4	75.4% ± 5.0	62.5% ± 6.6	59.4% ± 3.0	54.9% ± 2.8	58.2% ± 3.0
	30% Solid	65.1% ± 1.8	70.8% ± 1.9	61.8% ± 0.3	54.4% ± 0.4	52.0% ± 4.2	49.3% ± 0.1
	35% Solid	66.0% ± 2.4	68.5% ± 2.8	56.2% ± 4.5	55.1% ± 1.6	47.7% ± 0.8	46.7% ± 1.0

Note: All enzymes worked at their optimal condition; Liquefaction (a) Techzyme Q-Add at 93 °C pH 5.6, (b) Alpha Extra at 93 °C pH 5.6, (c) Spezyme® Alpha at 84 °C pH 5.7, and saccharification (d) GC 147 at 61.5 °C pH 4.2, (e) GA extra at 52.5 °C pH 4.7.

The heat map analysis shown in Fig. 4 also confirmed that the enzyme matching between AIII/GI, which was Techzyme Q-Add amylase for very high gravimetric (VHG) liquefaction subsequently saccharification with GC 147 glucoamylase, achieved highest TRS yield for all solid contents investigated. Matching between AI/GII which was Spezyme® Alpha and GC Extra was not suitable for VHG liquefaction and saccharification since the solid content above 25% to 35% dramatically decreased the TRS yield compared to other matching designs tested. From enzyme matching design, therefore, at 2 h liquefaction by Techzyme Q-Add and 48 h saccharification by

GC 147 were suitable for dry cassava root hydrolysis at 35% solid to achieve the highest TRS concentration. The highest TRS yield from liquefaction by Techzyme Q-Add and subsequently saccharification by GC 147 was in good agreement with the highest actual activity of enzyme (U g^{-1} protein) compared with other enzymes tested as aforementioned in enzyme characterization section.

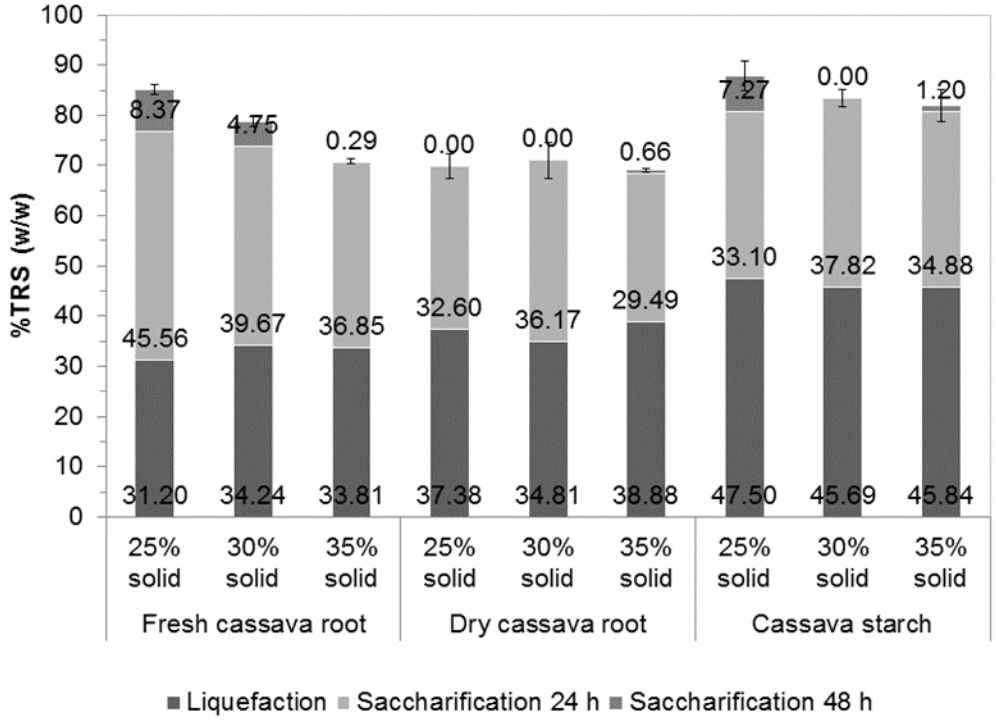

Fig. 4. TRS yields from fresh cassava root, dry cassava root, and cassava starch hydrolysis by Techzyme Q-Add liquefaction (2 h) and subsequent 24 h and 48 h saccharification by GC 147 when solid content was 25%, 30%, and 35%.

VHG Liquefaction and Saccharification of Fresh Cassava Root, Dry Cassava Root, and Cassava Starch

The best matching enzymes from the previous experiment, Techzyme Q-Add alpha-amylase and GC 147 glucoamylase, were selected. Fresh cassava root, dry cassava root, and cassava starch were liquefied by Techzyme alpha-amylase for 1 h and 2 h, and subsequently saccharified by GC 147 glucoamylase for 24 h and 48 h. TRS yield based on dry substrate weight is shown in Fig. 5.

The highest TRS yield from cassava starch liquefaction and saccharification was 87.9% w/w. The TRS yield from fresh cassava root hydrolysis noticeably decreased when the solid content increased. In contrast, the TRS yield from dry cassava root was constant *i.e.* 68.3, 70.5, and 69.0% w/w from 25%, 30%, and 35% solid, respectively. At 35% solids, TRS yield of dry cassava root was similar in degree to fresh cassava root. The highest TRS concentration was obtained from 35% solid liquefaction and saccharification. TRS concentration from 35% solids hydrolysis of cassava starch, fresh cassava root, and dry cassava root were 286.8, 249.4, and 241.1 g/L, respectively. The results corresponded to the amylose content found in the solid residue during liquefaction and saccharification, as demonstrated in Fig. 6.

The amylose content decreased as hydrolysis time increased. For cassava starch, the amylose content in the solid residue was rapidly decreased during 2 h of liquefaction, and it could not be quantified, as there was no solid residue left during the saccharification step. In the case of cassava root, the amylose content was rapidly decreased during 2 h liquefaction and slightly decreased for 48 h of saccharification. At the end of the saccharification process, 6.3% and 6.8% amylose content were found in fresh and dry cassava root, respectively.

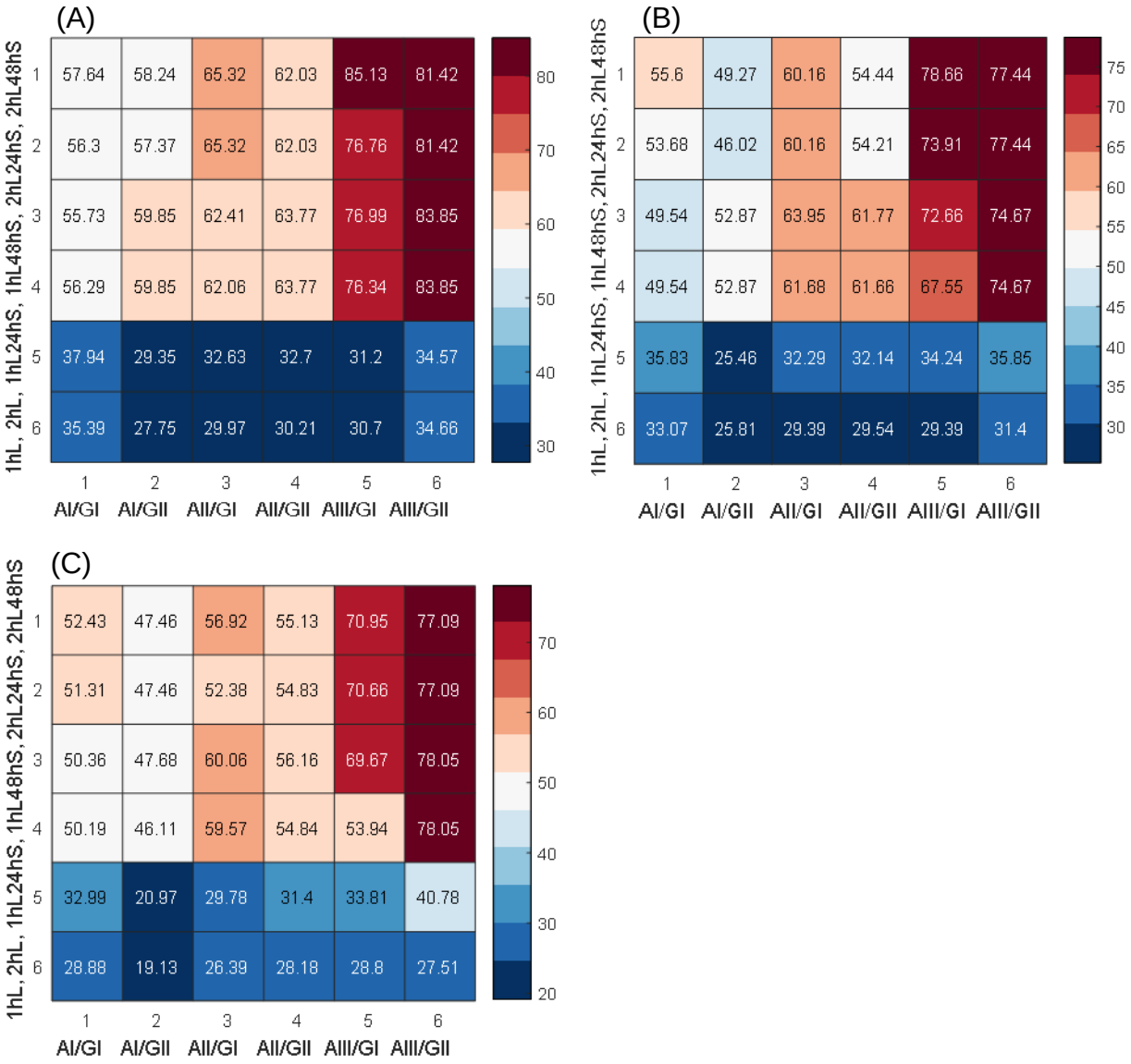

Fig. 5. Heat map of % TRS yield of fresh cassava root liquefaction for 1 h (1 hL) and 2 h (2 hL) and subsequently saccharification for 24 h (24 hS) and 48 h (48 hS) when using (A) 25, (B) 30, and (C) 35% solid content. Amylase enzymes used for liquefaction namely; AI = Spezyme® Alpha, AII = Alpha Extra, and AIII = Techzyme Q-Add, and glucoamylase used for saccharification namely; GI = GC 147 and GII = GC Extra

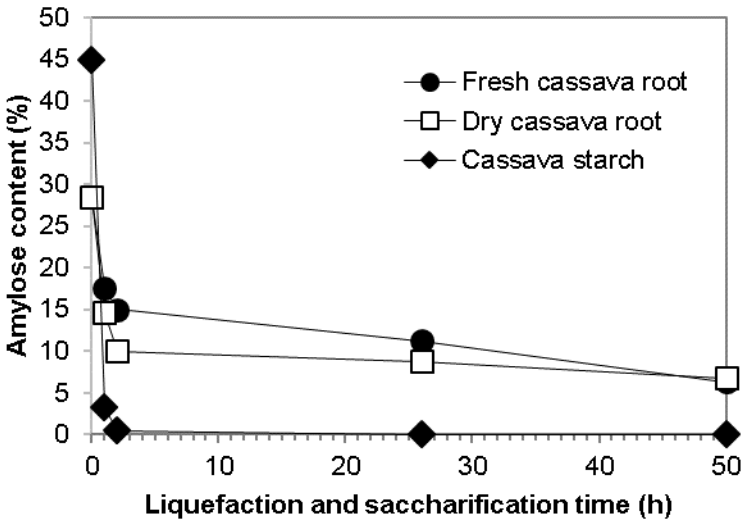

Fig. 6. Amylose content of solid residue during 2 h liquefaction by Techzyme Q-Add (alpha-amylase enzyme) and 48 h saccharification by GC 147 (glucoamylase enzyme) of fresh and dry cassava root at 35% solid content.

From SEM image analysis (Fig.7), the native milled cassava root contained most of starch granules attached to the fibers (Fig. 7(a)). When the cassava root underwent the liquefaction for 1 h at 93°C with Techzyme Q-Add, starch granules disappeared as they are liquefied in the presence of amylase (Fig.7(b)). The longer time the liquefaction took place, the more starch paste reduced and the fiber appeared (Fig. 7(c)). Similar phenomena occurred for the saccharification of cassava root (Fig. 7(d) and 7(e)) as the starch and oligomers were hydrolyzed to sugars by amylase and glucoamylase. Therefore, fiber, which is cellulose, remained intact. There was no significant difference of physical changes between fresh and dry cassava root (Fig. 7(f) to 7(i)) during liquefaction and saccharification by SEM image analysis.

Results of analysis of constituents in hydrolysate of fresh cassava root liquefaction by Techzyme Q-Add for 2 h and saccharification by GC 147 for 48 h are shown in Table 4. TRS concentrations were 213.4, 237.5, and 249.4 g/L for 25, 30, and 35% solid, respectively. Major components in hydrolysate were glucose (91.73 to 105.49 g/L) and respective degrees of dextrins (25.4 to 36.9 g/L), maltose (3.8-7.6 g/L), maltotriose (0.30 to 0.97 g/L), and xylose (0 to 2.73 g/L). When compared with TRS concentration, it was postulated that unquantified substances in hydrolysate were insoluble oligosaccharides.

Table 4. TRS and Glucose Concentration from Fresh Cassava Root Hydrolysis by Techzyme Q-Add Liquefaction (2 h) and Subsequent 48 h Saccharification by GC 147

Sample	Sugars Concentration (g/L)					
	TRS	Glucose	Xylose	Dextrins	Maltotriose	Maltose
25% Solid	213.4 ± 3.2	91.73 ± 6.12	1.41 ± 1.99	30.8 ± 5.7	0.30 ± 0.06	3.8 ± 0.3
30% Solid	237.5 ± 1.6	105.49 ± 0.72	0.00 ± 0.00	36.9 ± 23.7	0.97 ± 0.94	4.4 ± 2.1
35% Solid	249.4 ± 0.2	100.43 ± 2.14	2.73 ± 1.55	25.4 ± 2.6	0.31 ± 0.02	7.6 ± 0.8

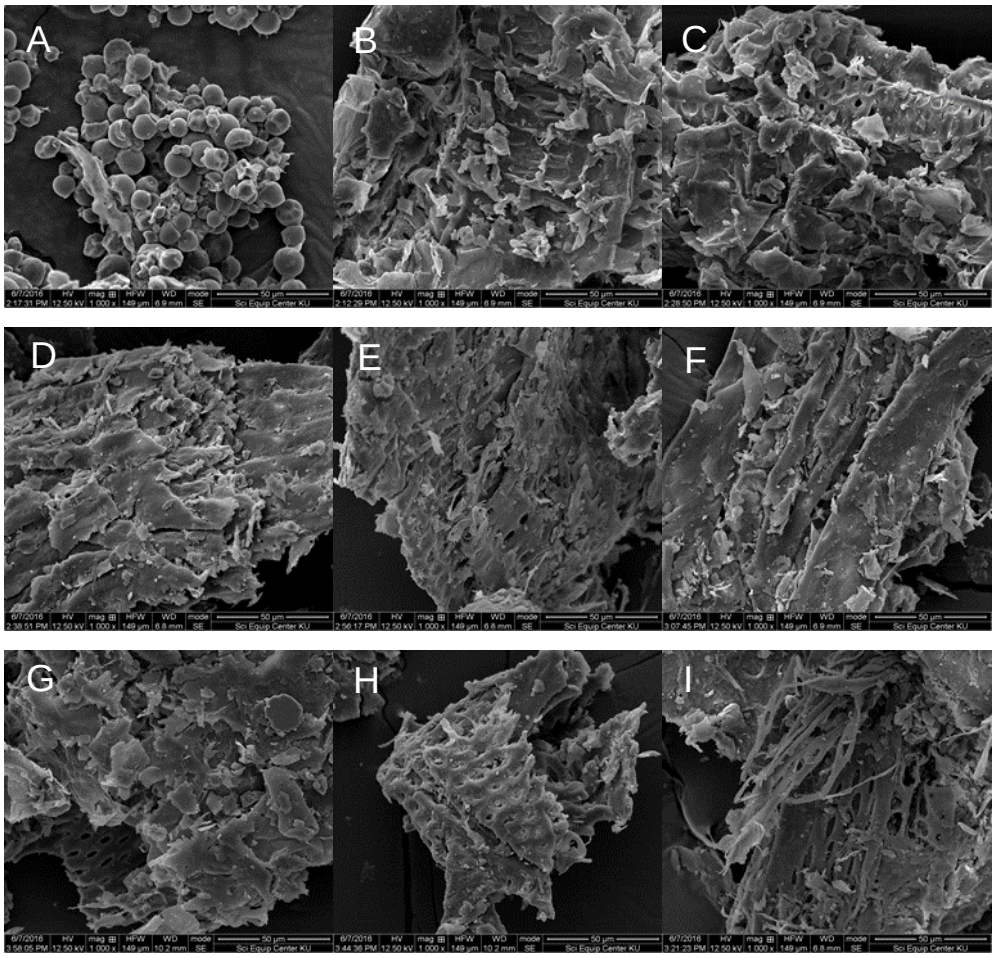

Fig. 7. SEM micrographs (1000×) for (A) controlled native milled cassava root, fresh cassava root samples namely (B) 1 h liquefaction, (C) 2 h liquefaction, (D) 2 h liquefaction/ 24 h saccharification, (E) 2 h liquefaction/ 48-h saccharification, and dry cassava root samples namely; (F) 1 h liquefaction, (G) 2 h liquefaction, (H) 2 h liquefaction/ 24 h saccharification, (I) 2 h liquefaction/ 48 h saccharification, when Techzyme Q-Add was used for liquefaction and GC 147 was used for saccharification.

Effect of Solid Content on Batch Ethanol Fermentation of Dry Cassava Root by SLSF and SLSSF System

The batch ethanol fermentation by the SLSF and SLSSF systems at 7.5, 10.0, 15.0, 25.0, and 35.0% solid of dry cassava root by *S. cerevisiae* TISTR 5606 was carried out at 30 °C for 72 h at 200 rpm. As shown in Fig. 8, the initial TRS concentration from SLSF was substantially higher than that of the SLSSF system in which glucoamylase was simultaneously added with *S. cerevisiae* inoculation. SLSSF produced slightly greater ethanol for all initial solid contents of dry cassava root except for 15% solid content where SLSF gave better ethanol concentration compared with SLSSF. However, TRS consumption during fermentation was not significantly different for all solid contents.

Table 5. Total Reducing Sugar, Sugar Composition, Acetic Acid, and Ethanol Concentration by *S. cerevisiae* TISTR 5606 at Different Solid Contents

Sample	Solid Content (%)	Concentration (g/L)											
		TRS			Dextrin			Maltotriose			Maltose		
		0 h	24 h	72 h	0 h	24 h	72 h	0 h	24 h	72 h	0 h	24 h	72 h
SLSSF	7.5	40.3	3.7	4.5	18.5	5.2	6.3	6.0	0.6	0.5	10.2	1.6	0.5
	10.0	60.5	5.4	3.2	21.1	5.1	0.0	1.3	0.0	0.3	13.6	2.4	0.6
	15.0	97.2	10.2	4.5	33.4	6.9	5.9	9.0	0.8	0.5	19.5	3.0	0.6
	25.0	159.2	26.0	7.3	51.8	9.1	0.0	7.0	1.0	0.2	32.9	5.0	1.3
	35.0	188.1	46.1	8.9	63.1	13.9	0.0	0.0	1.5	0.3	35.3	5.9	1.6
SLSF	7.5	52.9	4.5	5.5	12.3	12.3	12.4	0.0	0.0	0.0	3.6	3.6	0.1
	10.0	82.4	6.3	6.8	14.4	14.5	15.0	0.0	0.0	0.0	3.4	4.4	0.2
	15.0	126.1	10.6	9.9	17.3	18.5	20.3	0.6	0.0	1.4	2.9	7.5	2.2
	25.0	214.4	13.0	16.4	20.2	20.7	20.7	1.6	2.2	1.4	2.2	7.5	2.2
	35.0	256.9	42.9	20.2	21.1	23.6	22.7	2.2	3.1	2.1	3.0	10.6	3.1

Sample	Solid Content (%)	Concentration (g/L)															Ethanol Yield, Yp (g EtOH/g TRS)
		Glucose			Xylose			Arabinose			Acetic Acid			Ethanol			
		0 h	24 h	72 h	0 h	24 h	72 h	0 h	24 h	72 h	0 h	24 h	72 h	0 h	24 h	72 h	
SLSSF	7.5	17.7	3.1	0.0	0.8	0.0	0.0	0.7	0.9	0.7	4.7	3.7	3.2	0.0	5.2	5.2	0.146
	10.0	27.7	4.6	0.0	1.1	0.0	0.0	0.9	1.3	0.9	4.4	4.1	3.1	0.0	8.1	8.2	0.143
	15.0	33.6	7.7	0.0	1.6	0.0	0.6	1.4	1.6	0.9	4.1	3.3	2.4	0.0	11.4	12.1	0.131
	25.0	57.4	19.2	0.0	2.7	0.0	0.0	2.2	3.1	2.0	4.1	3.1	2.3	0.0	20.5	21.6	0.142
	35.0	78.7	34.3	0.5	3.5	0.0	1.1	2.8	4.0	3.4	4.0	3.0	3.2	0.0	26.2	27.3	0.152
SLSF	7.5	33.7	0.6	0.0	1.6	0.0	0.0	0.7	0.9	0.6	4.3	3.7	3.3	0.0	5.8	4.6	0.119
	10.0	46.1	0.2	0.0	2.1	0.7	0.0	1.0	1.1	0.8	4.3	3.5	3.4	0.0	7.8	7.0	0.102
	15.0	67.3	1.4	0.0	2.8	0.0	1.5	1.4	1.8	2.3	4.2	3.1	4.1	0.0	11.7	19.1	0.165
	25.0	96.4	0.6	0.0	4.6	2.3	1.6	2.5	3.1	2.6	4.2	3.2	4.2	0.0	19.5	16.8	0.097
	35.0	107.4	15.8	0.0	5.3	2.0	1.4	3.0	4.3	3.4	4.1	3.1	3.4	0.0	25.7	25.5	0.120

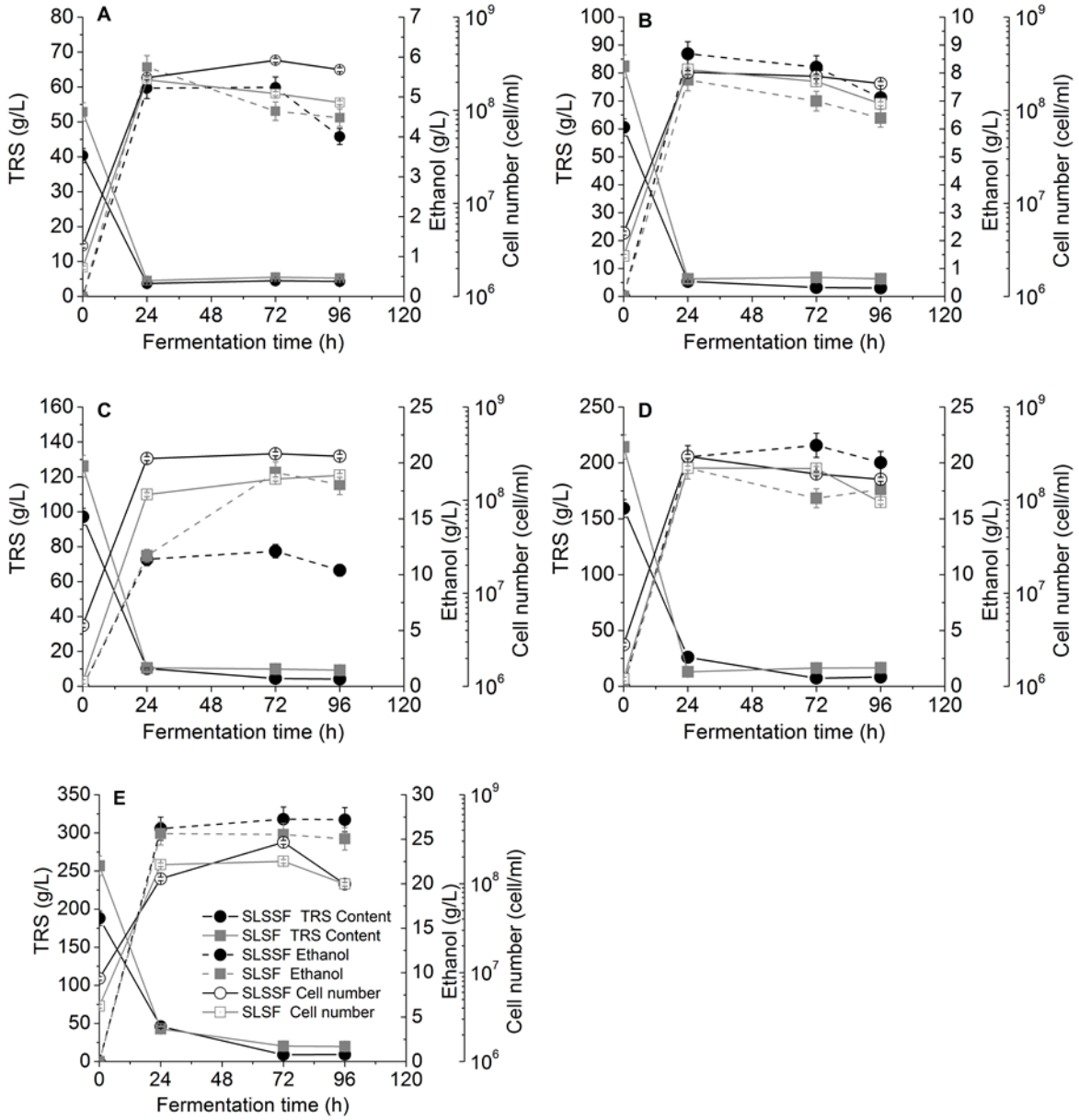

Fig. 8. Ethanol and TRS concentration during batch ethanol fermentation using SLSF and SLSSF system by *S. cerevisiae* TISTR 5606 for 96 h when initial dry cassava starch contents were (A) 7.5, (B) 10.0, (C) 15.0, (D) 25.0, and (E) 35.0% solid.

When constituents in the fermentation system were taken into consideration (Table 5), the accumulation of dextrins, maltose, and maltotriose was observed in the SLSF system for all samples taken at 0 h, 24 h, and 72 h. In contrast, SLSSF gave a larger amount of initial dextrins concentration at 0 h and some contents of maltotriose as well as maltose. However, all dextrins, maltotriose, and maltose were consumed, and only trace amounts were detected at the end of fermentation (72 h) with higher ethanol yield achieved compared to the SLSF system. The production of ethanol in the SLSF system ceased after 24 h of fermentation, as shown in Fig. 8. Furthermore, acetic acid was noticeably increased

until the end of the fermentation period, while the ethanol fermentation progressed until 72 h in SLSSF and thus ended up with a higher ethanol yield for the SLSSF system. Nevertheless, the highest ethanol yield was obtained from the SLSF system when the solid content was 15%. The solid content below or greater than 15% was not suitable for *S. cerevisiae* TISTR 5606 to grow and produce ethanol, which led to lower Y_p than that of SLSSF. Another reason was the significant loss of GC 147 activity in SLSSF process at 37 °C, since the optimum temperature of GC 147 is 61.5 °C. From the activity assay, the results indicated that the decrease of saccharification temperature of GC 147 from 61.5 °C to 37 °C reduced its activity from 11890 to 2183 U μL^{-1} or 3601 to 661 U g^{-1} protein. This is the major limitation of using thermotolerant enzyme for simultaneous saccharification and fermentation when the optimum temperature of enzyme for saccharification is substantially different from the temperature suitable for microorganism growth.

After the gelatinization of the starch in the mash prepared from cassava root, starch degradation to glucose was performed by two groups of enzymes namely 1) α-amylases acting on the cleavage of α,1-4 glycosidic bond to liquefy starch to dextrins in an endo-manner; and 2) glucoamylases attacking α,1-4 and α,1-6 glycosidic bonds at the non-reducing ends to saccharify the dextrins to glucose (Labout 1985). Among all α-amylases tested, Spezyme® Alpha gave the highest liquefaction yield, expressed as TRS, compared with others (Fig. S1) although the liquefaction temperature was 84 °C, which was only slightly higher than the gelatinization temperature of starch relative to 93 °C of Techzyme Q-Add and Alpha Extra. An increase of liquefaction duration of Spezyme® Alpha from 1 h to 2 h significantly enhanced liquefaction yield for all α-amylases investigated. Spezyme® Alpha is a high performance thermostable amylase and fast acting thinning enzyme suitable for hydrolyzing starch to dextrins in the VHG system when solid content is > 300 g/L, which has been used in thick root slurry within 30 min at its optimal temperature of 84 to 90 °C (Johnson *et al*. 2009).

To enhance the starch hydrolysis efficiency, several approaches have been applied to optimize product yield (Bao *et al.* 2011), to reduce the processing time, or to improve energy utilization efficiency (Johnson *et al.* 2009). However, in the present study, when the synergistic effect of the enzyme matching approach between different α-amylases and glucoamylases was considered, the heat map analysis results (Fig. 4) suggested that a combination of utilizing Techzyme Q-Add for liquefaction and GC 147 for saccharification could substantially enhance saccharification yield up to the highest amount of TRS during 24 h of the saccharification period (Table 3). A previous work reported that most of the commercial glucoamylases available in the market contain some amount of α-amylase released from specific starch hydrolyzing microorganisms during growth (Labout 1985). Another reason for synergistic action between Techzyme Q-Add and GC 147 was the suitable ratio of glucoamylase to α-amylase. There was a report stating that an increased ratio of glucoamylase to α-amylase significantly enhanced glucose production from raw corn (Arasaratnam and Balasubramaniam 1993). Compared between GC 147 and GA extra, GC 147 has a better thermo-tolerant property as it withstands high saccharification temperatures of up to 61.5 °C, while GA extra is suitable for operation at 52.5 °C. In the VHG system of fresh tubers mash, the process of liquefaction, saccharification, and fermentation is more complicated than the fermentation of the easily accessible sugars and starch due to the high viscosity of the mash. Highly viscous mash causes difficulty of mixing, causing accumulative CO_2 and ethanol. At a high temperature, significant reduction of viscosity of tubers mash was observed. Moreover, an improvement of mass

transfer by diffusion and mixing phenomena is greatly facilitated in such an elevated temperature system. Apart from temperature, GC 147 could also be used at lower pH levels or higher acidic conditions in which starch hydrolysis efficiency is considerably enhanced due to proton or H+ catalytic effect on α,1-4 glycosidic linkage break down. Starch is a polyacetal that is naturally sensitive to protonic acids, which readily catalyze its hydrolysis. In acidic conditions, in which a very small amount of weak organic acids is present, amylose helix resides in the inclusion complex form and substantially decreases in the viscosity of starch gels and starch stability (Tomasik and Schilling 2004). A lengthened period of saccharification from 24 to 48 h could significantly enhance saccharification yield only when high solid content at 35% is used. For other solid contents (25 and 30%), 2 h liquefaction by Techzyme Q-Add and subsequent 24 h saccharification of fresh cassava root by GC 147 was enough to achieve highest TRS yield based on cassava weight.

In contrast, the matching of other enzymes gave significantly lower yield of TRS based on fresh cassava root hydrolyzed especially AI/GII matching, for which it was Spezyme® Alpha/GC Extra that gave very low TRS yield at 35% solid content (Fig. 4 (c)). The reason was possibly due to high osmotic pressure in the VHG system that influenced enzyme activity and it was found that increased amylase and glucoamylase doses could considerably enhance the hydrolysis yield, leading to higher TRS yield (Puligundla *et al*. 2014). For fresh cassava root hydrolysis, hydrolysis rate could also be limited by the amount of fiber and the structure of starch granules and fiber. This required some extent of cellulase activity for the liquefaction step to release the starch granule from the fiber matrix. Moreover, it has been reported that several structural factors that could limit hydrolysis of starch included diffusion of enzyme molecules, porosity of solid substrates, adsorption of enzymes onto solid substrates, and the catalytic situation (Colonna *et al*. 1992). Further study on mechanistic modeling and identification of each of the factors affecting hydrolysis efficiency could be possible.

Compared among fresh, dry cassava root and cassava starch, TRS yield from cassava starch was maximum. Respective degrees of TRS yield from liquefaction and subsequent saccharification of fresh and dry cassava root were observed as shown in Fig. 5. To increase TRS yield of cassava root, cell wall degrading enzyme complexes such as endoglucanase, exoglucanase, cellulose, pectinases, and xylanases were necessary (Poonsrisawat *et al*. 2014, 2016). Moreover, amylose content reduction had resistant starch attached to fiber, which led to incomplete starch hydrolysis (Fig. 4). One possible means to solve this problem is alkaline solubilization prior to complete hydrolysis (Tomasik and Schilling 2004).

The action of α-amylase on the amylose and amylopectin components of starch proceeds in two stages. Initially, a complete rapid degradation of starch into shorter oligosaccharides with varying chain lengths takes place. This initial step is the result of a random attack on the substrate by the enzyme. Typical of this breakdown is a rapid loss of viscosity and an increase in reducing sugar reactivity. The second stage involves a slow hydrolysis of the oligosaccharides, with the formation of glucose and maltose as the final products from amylose. Glucose, maltose, and limit dextrins containing the α-l,6 linkages are the final products from amylopectin. Table 4 demonstrates the ratio of dextrins (DP ≤ 10), maltotriose (DP3), maltose (DP2), glucose (DP1), and the rest, which were oligomers (DP > 10) in hydrolysate from 2 h liquefaction by Techzyme Q-Add and subsequent 48 h saccharification by GC 147 of fresh cassava root. The results are in good agreement with a previous study reporting that Maltotriose (DP3) and oligomers (DP4) appeared rapidly

after 1 h of liquefaction and further hydrolyzed to glucose (DP1) and maltose (DP2), which were products of amylose hydrolysis (Pasari *et al.* 1988). For amylopectin hydrolysis, maltose (DP2) and maltotriose (DP3) gradually occurred and further hydrolyzed after 10 to 12 h, while glucose (DP1) and dextrins (DP $\geq$ 4) were products after complete hydrolysis. However, the results were dependent on the enzyme source and performance, which needs to be investigated particularly.

For batch ethanol fermentation of cassava root hydrolysate, the SLSF system in which 2 h liquefaction by Techzyme Q-Add and subsequent 48-h saccharification by GC 147 prior to ethanol fermentation gave significantly higher ethanol yield based on TRS consumed when compared with SLSSF (Table 5). Accumulative dextrins, maltotriose, and maltose were found in substantial amounts in the SLSF system at 72 h of fermentation, especially when high solid contents (15, 25, and 35%) were used. This was mainly due to the slow growth rate of yeast when yeast cells are subjected to sugar concentrations that are too high which leads to high osmotic stress in fermentation broth (Reddy and Reddy 2006; Srichuwong *et al.* 2009; Watanabe *et al.* 2010). To solve the problem, simultaneous addition of glucoamylase enzyme after 2 h liquefaction and inoculation of yeast in the SLSSF system was proposed. In the present work, the ethanol yield from the SLSSF system was significantly enhanced compared with the SLSF system. Moreover, less accumulative sugars (dextrins, maltotriose, and maltose) were found at 72 h of fermentation, as shown in Table 5.

The growth rate and ethanol production rate of yeast were also observed in Fig. 8. For low solid content at 7.5% and 10% (Fig. 8(a) and 8(b)), TRS was totally consumed after 24 h of fermentation, and maximum ethanol concentration was also measured at the same time. With the solid content higher than 15% (15, 25, and 35%), slow TRS consumption was found (Fig. 8(c), 8(d), and 8(e)). At high solid content, as mentioned before, maximum TRS consumption was found at 24 h and gradually TRS utilization was observed until 72 h of fermentation, where maximum ethanol production was measured. Apart from the genetic engineering of yeast, some solution methods for enhancing ethanol yield and production rate, improving cell viability, and increasing yeast growth rate of the VHG system have been proposed in several previous works dealing with the addition of nitrogen sources such as ammonia, yeast extract, and ammonia ions (Reddy and Reddy 2006; Li *et al*. 2017) due to increased free amino nitrogen (FAN) and/or fatty acid concentration (Kawa-Rygielska and Pietrzak 2014). Bioprocess engineering technology could also be applied to enhance ethanol yield in the VHG system, including implementation of the fed batch feeding pattern (Unrean and Khajeeram 2016; Zhang and Zhu 2017), application of separated-tank bioreactor or membrane bioreactor (Wang *et al*. 2013; Najafpour 2015), utilization of SSF with enzymes or substrate feeding control strategy (Olofsson *et al*. 2010), *etc*. In such processes, the balance of substrate consumption and ethanol production was well done. In addition, control of temperature, pH, and aeration or atmospheric gas in the bioreactor was proposed to facilitate the control of the redox potential of substrate degradation and assimilation in ethanol fermentation in the VHG system (Ingledew and Lin 2011). In the present study, the SLSSF system gave higher ethanol yield compared with the SLSF system and moreover shortened the overall liquefaction (2 h), saccharification (48 h), and fermentation (24 h) of the HVG system from 74 h to only 26 h to achieve the highest ethanol yield and the highest ethanol productivity.

CONCLUSIONS

1. In the very high gravity liquefaction and saccharification of cassava root, the enzyme matching design approach was successfully improved in reducing sugar yield.
2. For ethanol fermentation, the SLSSF system showed substantially better ethanol production yield compared with the SLSF system in which initial sugar concentration was very high. The results suggested that SLSSF could effectively shorten the time course of the entire process for liquefaction, saccharification, and fermentation (74 h) to only 26 h for similar ethanol production yield.
3. To enhance ethanol yield from the VHG system, the biotechnological approach to reduce osmotic stress and increase yeast cell availability, as well as boost up cell growth rate, such as the addition of assimilable nitrogen sources, design of substrate, enzyme feeding strategy, and controlling process parameters *e.g.* pH, temperature, aeration, *etc*. are promising means.
4. An enhancement of ethanol production efficiency using SSF process could also be accomplished by reducing the gap between the optimal temperature of saccharifying enzyme and the optimal temperature of yeast growth.

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