Single-Step Ethanol Production from Raw Cassava Starch Using a Combination of Raw Starch Hydrolysis and Fermentation, Scale-Up from 5-L Laboratory and 200-L Pilot Plant to 3,000-L Industrial Fermenters

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Abstract

Background: A single-step ethanol production is the combination of raw cassava starch hydrolysis and fermentation. For the development of raw starch consolidated bioprocessing (CBP) technologies, this research work was to investigate the optimum conditions and technical procedures for the production of ethanol from raw cassava starch in a single step. This resulted high yields and productivities of all the experiments from the laboratory, the pilot, through the industrial scales. The yields of ethanol concentration are comparable with those in the commercial industries that use molasses and hydrolyzed starch as the raw materials.

Results: Before single-step ethanol production, the studies of raw cassava starch hydrolysis by a granular starch hydrolyzing enzyme, StargenTM002, were carefully conducted. It successfully converted 80.19% (w/v) of raw cassava starch to glucose at a concentration of 176.41 g/L with a productivity of 2.45 g/L/h when the raw starch was pretreated at 60 °C for 1 h with 0.10% (v/w dry starch basis) of Distillase ASP before hydrolysis. A single-step ethanol production at 34 °C in a 5-L fermenter showed that *S. cerevisiae* (Fali, active dry yeast) produced the maximum ethanol concentration, p of 81.86 g/L (10.43% v/v) with a yield coefficient, $Y_{p/s}$ of 0.41 g/g, a productivity or production rate, r_p of 1.14 g/L/h with an efficiency, Ef of 71.44%. The scale-up experiments of the single-step ethanol production using this method, from the 5-L fermenter to the 200-L fermenter and further to the 3,000-L industrial fermenter were successfully achieved with essentially good results. The p, $Y_{p/s}$, r_p, and Ef values of the 200-L scale were 80.85 g/L (10.23% v/v), 0.41 g/g, 1.12 g/L/h and 72.47% , respectively ; of the 3,000-L scale were 70.74 g/L (9.01% v/v), 0.34 g/g, 0.98 g/L/h and 59.82% , respectively. Because of using raw starch, the major by-products of all the three scales were very low; glycerol lactic acid and acetic acid, in ranges of 0.94-1.14%, 0.046-0.052%, 0-0.059% (w/v), respectively, where are less than those values in the industries.

Conclusions: This single-step ethanol production using a combination of raw cassava starch hydrolysis and fermentation of the three fermentation scales here is practicable and feasible for the scale-up of industrial production of ethanol from raw starch.

Keywords: bioethanol; single-step ethanol production; raw cassava starch, hydrolysis, fermentation, pilot scale, industrial-scale

1. Background

Bioethanol is an alternative energy source to replace the utilization of those fuels. Bioethanol is an attractive alternative fuel because it is eco-friendly renewable bio-based resource contributing to the reduction of fuel emissions, effects of climate change, and negative environmental impacts generated by the worldwide utilization of the petroleum oil [1-3]. Cassava is the major energy crop and one of the renewable resources that is utilized for bioethanol production. Cassava starch can be used at large scales to produce ethanol for the tropical countries where Thailand is one of the largest cassava producers in the world [4]. Furthermore, it is a cheap, clean, non-toxic, and renewable carbohydrate carbon source, and available in very abundance, thus is widely used as an important feedstock for industrial applications especially in the ethanol production [5]. Therefore, bioethanol production from starch has been extensively researching [6] and still need more developments for more effective industrial productions with higher efficiencies.

Our research work is herein pertinent with the "Recent Advances" [7] in June 2020 addresses for starch-to-ethanol conversion have provided a platform for the development of raw starch consolidated bioprocessing (CBP) technologies. Several proof-of-concept studies identified improved enzyme combinations, alternative feedstocks and novel strains [8, 9] for evaluation and application under fermentation conditions. However, further research efforts are required before this technology can be scaled-up to an industrial level. In their review, different CBP approaches are defined and discussed, also highlighting the role of enzymes for a supplemented CBP process. Various achievements of amylolytic *Saccharomyces cerevisiae* strains [8, 9] for CBP of raw starch and the remaining challenges that need to be tackled / pursued to bring yeast raw starch CBP to industrial realization are described in [7]. The conventional starch liquefaction and saccharification processes are energy-intensive, complicated, and not environmentally friendly. Raw starch-digesting glucoamylases are capable of directly hydrolyzing raw starch to glucose at low temperatures, which significantly simplifies processing and reduces the cost of producing starch based products [10].

At present, the conventional large-scale ethanol production from starch is a batch process [5] comprises three steps: liquefaction by alpha-amylase, which reduces the viscosity of the starch and fragment the starch into small sized chains, followed by saccharification whereby the liquefied starch is hydrolyzed to fermentable sugar using glucoamylase. Finally, the glucose is fermented into ethanol by

yeast cells [11, 12]. In the process to convert starch into ethanol, starch granules must be gelatinized and liquefied at high temperature before saccharification and fermentation [13]. The conventional enzymatic liquefaction and saccharification of starch has many disadvantages. It requires a high-energy input [14] including the enormous amounts of steam and efficient water-based cooling system to bring down the temperature for fermentation [15], thus increasing the production cost of starch-based ethanol. Besides the conventional process of ethanol production, simultaneous saccharification and fermentation (SSF) process has been widely used [16]. After liquefaction, saccharification is performed simultaneously with fermentation. This process uses glucoamylase and free cells of yeast at the same time in a single fermenter. The advantages of SSF process include reduction of cost because less equipment and fermentation time required, resulting in higher ethanol concentration and productivity [17, 18]. However, SSF processes also have significantly impacted energy consumption because liquefaction takes place at high temperatures. Therefore, processes to reduce high energy consumption are required. If starch hydrolysis step at gelatinization temperature was avoided the costs due to the high energy consumption of starch-based ethanol in the manufacturing process can be save with 30-40% [19]. To overcome the high-temperature-cooking fermentation in industrial ethanol production from cassava starch, we have constructed a single-step ethanol production process (simultaneous raw starch hydrolysis and ethanol fermentation in a single fermenter). This process not only the yeast fermentation but also includes the hydrolyzation of raw cassava starch at low temperature by the addition of raw starch-digesting enzymes at the initial stage, which significantly decreases the energy consumption of overall process and the operation cost resulted from multistage process of ethanol production. Moreover, the low temperature fermentation has been used in pilot-plant scale and industrial-scale production without contamination by bacterial cells, and yielded the fermentation efficiency similar to that of the conventional fermentation [11]. The other direct advantage of single-step process is kept low concentration of glucose during fermentation which decrease inhibition effect of glucose on the enzymes and yeast activity.

In the present study, we intend to combine both raw cassava starch hydrolysis and ethanol fermentation in a single process step for reducing high energy consumption by utilizing enzymes which are capable of hydrolyzing raw cassava starch under the fermentation conditions and saving the operation cost by combination of raw cassava starch hydrolysis and fermentation in a single fermenter. This study aimed to optimize the hydrolysis of raw cassava starch by StargenTM002 and to develop a commercially practical single-step ethanol production process using raw cassava starch as a raw material at laboratory, pilot, and industrial scales.

2. Results and Discussion

2.1 Optimization of Enzyme StargenTM002 Conditions for Raw Cassava Starch Hydrolysis

The formation and percent conversion of glucose from raw cassava starch hydrolysis by varying the enzyme StargenTM002 concentration, temperature, and pH, for those optimal values are shown in Table 1. Considering to the enzyme dosages, it was found that the highest glucose concentration was achieved by using StargenTM002 at a concentration of 0.3% (v/w ds). A 200 g/L of raw cassava starch with the enzyme dosage at a concentration of 0.3% yielded the glucose at a concentration of 73.78 g/L with 33.54% (w/w) degree of conversion. In order to assess the effect of temperature on raw cassava starch hydrolysis, the hydrolyzations were carried out at 30, 35 and 40 °C. There were the increases in the quantities of glucose released and higher percentages of conversion of raw starch to glucose when the temperature of starch slurry was increased from 30 °C to 40 °C. The hydrolysis at 40 °C generated the highest glucose concentration of 133.27 g/L with 60.58% (w/w) degree of conversion. Further, the pH values of raw starch slurry from pH 3.0-7.0 were verified for study its effect on raw starch hydrolysis. The results showed that the glucose concentrations and the percentages of starch conversion were increased when the pH values of starch slurry were decreased from 7.0 to 3.0. The starch hydrolysis by StargenTM002 at pH 3.0 yielded the maximum glucose concentration of 114.39 g/L with 52% (w/w) of starch conversion. However, in this experiment, we concerned that the pH value below 4.0 could be a negative effect on yeast cell growth and fermentation activities. The pH is accepted as one of the most important parameters influencing yeast cell growth and fermentation activities. It is known that low initial pH values causes chemical stress on yeast cells and showed affecting the accumulated mass loss, decreasing the composition rate of sugar, increasing the final concentration of glycerol, and decreasing the final concentration of ethanol [20, 21]. Several studies investigating the influence of pH on *S. cerevisiae* fermentations have been published. For examples, a pH at 4.5 gave the highest ethanol production from *S. cerevisiae* [22], a pH below 3.5 lead to reduce yeast viability and vigor as well as lower ethanol yield [23], the optimal pH values for yeast growth can vary from 4.0-6.0, depending on yeast strains, and the decrease in ethanol production was observed when the initial medium pH was at 3.0 [24], and The pH is considered an important factor for the survival and growth of yeasts. It affects on the permeability of the cell membrane and on the enzymes that are active in degrading the substrate [25]. Therefore, in our further studies, the pH value at 4.0 was used as the optimum pH on raw starch hydrolysis and ethanol fermentation.

2.2 Raw Cassava Starch Pretreatments and Hydrolysis

The formation and the conversion degree of glucose from raw cassava starch hydrolysis by enzyme StargenTM002 at the optimum conditions for 72 h are shown in Table 4. It was found that the hydrolysis

raw cassava starch at pH 4.0 and 40 °C for 72 h with StargenTM002 at a concentration of 0.3% (v/w ds) generated the glucose at a concentration of 150.83 g/L with 68.56% (w/w) degree of conversion. This result indicated that it was incomplete hydrolysis. Consequently, the pretreatment of raw cassava starch which influences the StargenTM002 activity is an interesting strategy for increasing the hydrolysis capability.

Table 2 shows the glucose content and degree of conversion of starch to glucose from heat, enzyme, and urea pretreatments of raw cassava starch for hydrolysis by StargenTM002 at the optimum conditions. The raw cassava starch was pretreated at a sub-gelatinization temperature of 60 °C for 1 h. After pretreatment, the starch slurry was hydrolyzed by StargenTM002 at the optimum conditions. The results showed that sub-gelatinization temperature pretreatment increased the raw cassava starch hydrolysis activity by StargenTM002. Compared with none the pretreatment, the heat pretreatment at 60 °C generated a higher glucose concentration of 159.90 g/L with 72.68% (w/w) degree of conversion.

Moreover, the pretreatment at sub-gelatinization temperature with enzyme Distillase ASP resulted an increase in glucose formation and degree of raw cassava starch conversion to glucose. The highest glucose concentration of 176.41 g/L with 80.19% (w/w) degree of conversion were sucessfully achieved when raw cassava starch was pretreated at 60 °C with 0.1% (v/w ds) of enzyme Distillase ASP before the step of StargenTM002 hydrolysis. However, the increase in Distillase ASP dosage for pretreatment did not significantly effect further raw starch hydrolysis by StargenTM002.

On the contrary, heat pretreatment with enzyme Distillase ASP plus urea at the concentrations of 1.0-3.0% (w/w sd) did not significantly effect raw cassava starch hydrolysis when compared with the pretreatment without urea. This result indicates that urea pretreatment does not improve raw cassava starch hydrolysis by StargenTM002. This studied result does not agree with [28] who reported that the combination of urea addition and sub-gelatinization temperature pretreatment greatly improved triticale and corn starch hydrolysis by breaking hydrogen bonds in starch molecules. The reasons for the difference between this result and those of [27] are unclear, but one of the reasons is the difference in starch structures of cassava to those of triticale and corn.

The morphological and microstructural changes of the pretreated and hydrolyzed raw cassava starch granules revealed by SEM are shown in Figure 1. From SEM micrographs, the surface of pretreated raw cassava starch granules at 60 °C with 0.1% (v/w ds) enzyme Distillase ASP for 1 h was smooth with few furrows and shallow depressions (Figure 1a). The hydrolyzation of raw cassava starch granules by StargenTM002 for 48 h resulted in degradation of most starch into glucose fermentable sugar. Many large enlarged surface holes were observed on the residual starch granule (Figure 1b).

Sub-gelatinization temperature pretreatment would allow the starch grains to swell and open up the pore on the granule surfaces [14] which increased the ability of Distillase ASP to hydrolyze starch granule surface resulting to increase surface area for StargenTM002 attack. The raw cassava starch hydrolysis by StargenTM002 was initiated from granule surface by size enlargement of existing holes [26]. Moreover, the roughened surface in hydrolyzed raw cassava starch granule might be due to uneven shortening of amylopectin molecules by the action of amylase enzyme [5]. Sub-gelatinization temperature pretreatment cause the starch granule to swell and Distillase ASP action increase the starch granules surface, resulting in the StargenTM002 to penetrate into the granule more extensively forms pits and channels during hydrolysis. The enzyme degraded the external part of starch granule by exo-corrosion as holes. These results were also in accordance with [27, 28] who reported that enzymatic corrosion occurred mainly from the cassava starch granule surface to the center. [14] also reported that rough surface and corroded granules were observed in hydrolyzed heat-treated starch compared to hydrolyzed native starch which displayed rough surface with limited erosion and fewer holes. Also [29] suggested that the enzymes are adsorbed on the surface of starch granule and induce holes on the surface where glucose is released.

The results from previously study showed an incomplete hydrolysis of raw cassava starch by StargenTM002. [30] reported that the degree of hydrolysis slightly increased after washing the residue starch and adding a fresh enzyme dosage. No rapidly hydrolysis was observed after remove the enzyme by washing and adding a new amylase solution. Therefore, it was assumed that the raw cassava starch hydrolysis was uncompleted due to the presence of a residue resistant starch. Using the X-ray diffraction patterns can differentiate between native and hydrolyzed starch by detection of change in crystallinity of granular starch [14, 26] who reported that the crystalline types of native and hydrolyzed cassava starch were not markedly changed. However, the crystalline peak of hydrolyzed starch become bigger compared with that of native starch. They concluded that the amorphous region of the granule was hydrolyzed more extensively than the crystalline region. Thus, in this study, the hydrolysis may primarily be occurs in the amorphous region of starch granules. When StargenTM002 hydrolyzed the starch granule, it could primary degrades the amorphous region. The crystalline structure may increase in the residue raw cassava starch.

2.3 Laboratory Single-Step Ethanol Production Using Combination of Raw Cassava Starch Hydrolysis and Fermentation

The single-step ethanol fermentations by *S. cerevisiae* (Fali) were conducted in 5-L fermenters each containing 4 L of fermentation medium composed of the pretreated raw cassava starch at a concentration of 200 g/L. Enzyme StargenTM002 and *S. cerevisiae* inoculants were added into the

fermentation medium at concentrations of 0.30% (v/w ds) and 0.10% (w/v), respectively. To study the effect of temperature on single-step ethanol production, the fermentations were performed at the temperatures of 30 °C, 40 °C, and without temperature control (with the initial temperature of 40 °C). The fermenters were agitated at the design speed of 200 rpm for 72 h along fermentations.

The temperature is one of the most important factors that effect the ethanol production. Figure 2 showed the effect of different temperatures on single-step ethanol production. Based on the result obtained, they were observed that the single-step ethanol fermentation at the temperature of 30 °C lead to the final ethanol concentrations of 70.92 g/L (9.03% v/v). At none temperature control (34 ±1 °C) the highest ethanol concentration was obtained at a concentration of 81.86 g/L (10.43% v/v). However, as the temperature increased to 40 °C, it generated the highest fermentation rate at the first 36 h and after that it slightly decreased with increasing time. The final ethanol at a concentration of 65.78 g/L (8.38% v/v) was obtained which was lower than those treatments of the 30 °C and non-control temperature. This finding is in agree with [24] who reported that the rate of enzyme catalyzed reaction in yeast cells increases with temperature up to a certain value and then the enzyme begins to denature resulting inhibition of yeast activities and significantly decreasing ethanol fermentation. Thus, a control of specific constant temperature was required for single-step ethanol production.

The glucose concentration of fermentation broth decreased with time for the temperature below 40 °C. In contrast to 40 °C the glucose rapidly decreased within the first 36 h, after that it was not utilized by yeast cells which consequently caused a result of final glucose accumulation in the system.

Though the *S. cerevisiae* growth profiles of all fermentation temperatures tended to approach the similar values except that at the temperature of 40 °C which was lower than those of 30 °C and non-control temperature. The results indicate that increasing the fermentation temperature resultes in decreasing growth and ethanol production by *S. cerevisiae*. The obtained data clearly show that the optimum temperature of around 34 °C at which both yeast and StargenTM002 work best together. If lower than 34 °C, the reaction rate of StargenTM002 was declined and if above this value the yeast cell growth and its activities would be inhibited.

The temperature directly affects metabolism and growth of yeast cells. At a wormer temperature, yeast cells show a rapid decline in viability at the end of fermentation while at a excessively high temperature, enzyme and membrane functions may disrupt which result to stuck fermentation [31] Moreover, heat stress causes a change of plasma membrane which reduces the levels of plasma membrane H^+-ATPs and transport system [32]. High temperature showed the inhibitory effect on ethanol production. The intracellular ethanol concentration was higher than the optimum level. Its accumulation within the cell was a consequence of the resistance to its diffusion through the cell wall

232 from inside to outside the cells [33]. It affects the plasma membrane of yeast cells resulting in altered
233 membrane organization and permeability [34]. Therefore, during ethanol fermentation, increasing both
234 temperature and ethanol concentration togetether acts to cause a reduction in growth rate, fermentation
235 rate, and cell viability. Together both heat and ethanol stress can cause reduction of metabolic activity
236 and eventually cell death.

237 In this study, we combined the enzymatic hydrolysis of raw cassava starch and ethanol
238 fermentation within a single stage. However, the optimum temperature at 40 °C for hydrolysis of raw
239 cassava starch was higher than that of fermentation. The design of operating temperature for single-step
240 ethanol production was very important. As mentioned before, the temperature at 40 °C was the
241 optimum for enzyme activity but it could reduce the growth and metabolism of *S. cerevisiae* whilst the
242 use of low temperature at 30 ° increased the yeast activity but reduce the hydrolysis rate of raw cassava
243 starch. As it has been explained above, the highest final ethanol concentration of 81.86 g/L (10.43% v/v)
244 with a productivity of 1.14 g/L/h and 0.41 g/g of ethanol yield coefficient were obtained under the
245 non-control fermentation temperature (34 ±1 °C). This result showed that the single-step ethanol
246 production by *S. cerevisiae* at approximately 34 °C provides the best compromised temperature that
247 enhanced the enzyme activity and promotes *S. cerevisiae* to produce ethanol at a high concentration.

248 Furthermore, it was interesting to note that the by-products, i.e. glycerol, lactic acid, abd acetic acid
249 were at only 1.14, 0.05, and 0.00% (w/v), respectively (Table 4). These major by-products in ethanol
250 production obviously were not produced, when using raw cassava starch as the raw material with
251 single-step fermentation. The advantages of this fermentation system is that the heat pretreatment at 60
252 °C for 1 h in the early process reduces acid-producing bacteria contamination resulting in a very low
253 amount of lactic acid and without acetic acid. This method also prevents the single-step ethanol
254 fermentation using raw cassava starch from other microorganism contamination.

255 ***2.4 Pilot and Industrial Scale Single-Step Ethanol Production Using Combination of Raw Cassava***
256 ***Starch Hydrolysis and Fermentation***

257 The main objectives of this session were to (i) evaluate the potential use of single-step ethanol
258 fermentation at larger scales, (ii) identify problems that were not significantly noticed at the laboratory
259 scale, and (iii) check the ethanol yield after single-step fermentation. According to the results from
260 laboratory-scale fermentation, the further single-step ethanol fermentations were conducted in 200-L
261 pilot and 3,000-L industrial fermenters. After enzyme additions and yeast inoculations, the single-step
262 ethanol fermentations using the combination of raw cassava starch hydrolysis and fermentation were
263 operated at the temperature of 34 °C with the same value as those of 5-L fermenters for 72 h with the
264 agitation rate at 125 rpm for 200-L fermenter and 55 rpm for 3,000-L fermenter. To keep the designed

scale-up paramater power input, $\bar{\varepsilon}_T$ of both scales constant, both agitation speeds equivalent to the same power input, $\bar{\varepsilon}_T$ of 0.10 W/kg).

The final ethanol at a concentration of 80.85 g/L (10.23% v/v) with 72.47 % efficiency was achieved when the single-step ethanol fermentation from raw cassava starch has been scaled-up to a 200-L fermenter (Figure 3 a). The maximum ethanol concentration in the 200-L fermenter was not significantly different from 81.86 g/L (10.43% v/v) of the 5-L laboratory fermentation. This result indicated that the operating conditions and its performance for this scale-up was significantly effective as that obtained from the 5-L fermenter. Furthermore, the performance of single-step ethanol fermentation in the 3,000-L industrial-scale fermenter was also studied. In Figure 3 (b) the production in the 3,000-L fermenter generated the maximum ethanol at a concentration of 70.74 g/L (9.10% v/v) with 59.82 % efficiency. It can be observed that there are similarities in the ethanol production and sugar utilization between both scales of fermentation. However, the latter showed a lower ethanol yield than the 200-L fermenter. It was indicated that the rate of starch hydrolysis by StargenTM002 was lower than that of glucose consumption by the yeast resulting in no enough glucose which causes a lower value.

A comparison of the results obtained from different scales of 5-L, 200-L, and 3,000-L fermentations was shown in Table 3 and Figure 3. The final ethanol concentration of 200-L fermenter was the same as that of the 5-L fermenter with the values of 80.85 and 81.86 g/L, respectively, corresponding to the same ethanol yield coefficients of 0.41 g/g with 71.44 and 72.47% eficiencies, and the productivities of 1.12 and 1.14 g/L/h at both fermentation scales, respectively. However, the final ethanol concentration in the 3,000-L fermenter was slightly less than that of the 5-L and 200-L fermenters, corresponding to less ethanol yield, fermentation efficiency, and productivity. This may be because of limitation of cooling system and heat transfer of the larger scale experimental operation.

The concentrations of by-products i.e. glycerol, lactic acid, and acetic acid the end of single-step ethanol productions remained very low at every scale of fermentation (Table 4). [22] have reported the minimum inhibitory concentrations of lactic and acetic acid were 0.80% and 0.05%, respectively. In this research work, the concentrations of both acid by-products remained lower than the minimum stressful values. It is clearly show that the values of ethanol content, glucose, glycerol, lactic acid, and acetic acid during single-step ethanol production using combination of raw cassava starch hydrolysis and fermentation were similar to those observed at the laboratory scale, which clearly indicated that no deviation was observed when scaled-up from the laboratory to the pilot and further to the industrial scales of operation. This indicates the scaleable potential and feasibility for a decision to a new route of industrial bioethanol production from raw starches.

3. Conclusions

The combination of raw cassava starch hydrolysis and fermentation is possible and practicable for single-step ethanol production. The granular starch hydrolyzing enzyme StargenTM002 showed high abilities for raw cassava starch hydrolysis. Under the optimum conditions, a 68.56% (w/w) of raw cassava starch was converted to glucose. Moreover, the pretreatment of raw cassava starch at 60 °C for 1 h with Distillase ASP (a blend of glucoamylase and pullulanase) greatly improved raw starch hydrolysis further by StargenTM002 which converted 80.19% (w/w) of raw cassava starch to glucose. For ethanol production from raw cassava starch, the highest ethanol concentration, productivity, and eficiency from single-step fermentation by *S. cerevisiae* (Fali, actively dried yeast) at 34 °C in a 5-L fermenter were achieved. This yielded the ethanol concentration of 81.86 g/L (10.43% v/v) with a productivity of 1.14 g/L/h and a yield coefficient of 0.41 g/g with 71.44% eficiency. The scale-up of the single step ethanol production using combination of raw cassava starch hydrolysis and fermentation from 5-L laboratory to 200-L pilot-scale and further to 3,000-L industrial fermenter was successfully undertaken. The three different working scales of fermenters showed little differences in a final ethanol concentration, productivity, yield, and fermentation efficiency. These results indicated that the single-step ethanol production using a combination of raw cassava starch hydrolysis and fermentation can be scaled-up to a larger capacity for the novel industrial production of bioethanol.

4. Materials and Methods

4.1 Materials

4.1.1 Microorganism

The manufacture active dried alcohol yeast, *Saccharomyces cerevisiae* (Fali), obtained from AB Mauri (Australia), was used in this study. This yeast strain could create the maximum ethanol yield exceeding 18% (v/v) or 12% (w/v) depending on fermentation procedures. Furthermore, it is extremely thermotolerant and has a wide fermentation temperature range of 25-40 °C. The dry yeast was re-hydrated in distilled water at 40 °C for 20 min prior to the addition into the single-step ethanol fermentation.

4.1.2 Materials

Cassava starch of the "Three Elephants" brand was obtained from Chorchiwat Industry Co., Ltd (Chonburi province, Thailand) with complements. After manufactured, freshly cassava starch was stored at dry and cool place in the laboratory store. The moisture content of the cassava starch in this study was 12 ± 1.0% and its pH was 5-7.

The commercial enzymes provided by DuPont Industrial Biosciences (previously known as Genencor, A Danisco Division) were utilized in this study. They were (i) StargenTM002 (granular starch hydrolyzing enzyme, containing *Aspergillus kawachi* α-amylase expressed in *Trichoderma reesei* and glucoamylase from *T. reesei*) and (ii) Distillase ASP (containing a blend of glucoamylase and bacteria pullulanase from *Bacillus licheniformis* and *T. reesei*). Properties of these commercial enzymes are presented in Table 5.

Table 5. Characterization of the commercial enzymes used in this study

Commercial enzymes (Types)	Optimal temperature (°C)	Optimal pH	Activity
StargenTM002 (Blending of alpha-amylase and glucoamylase)	20-40	4.0-4.5	570 GAU/g[a]
Distillase ASP (Blending of glucoamylase and pullulanase)	58-65	4.0-4.5	580 GAU/g

[a] A one Glucoamylase Unit (GAU), defined by DuPont, is the amount of enzyme that will liberate one gram of reducing sugars calculated as glucose per hour from soluble starch substrate under the conditions of the assay

4.2 Methods

4.2.1 Optimization of StargenTM002 Conditions for Raw Cassava Starch Hydrolysis

The 1-L volumes of starch slurry in the glass containers, each contained 20% (w/v) of raw cassava starch prepared in distilled water, were incubated in a water bath at 30 °C for 48 h, with continuous stirring at 100 rpm using an overhead stirrer. The StargenTM002 were added into the raw cassava starch slurries at the concentrations of 0.1-0.4% (v/w of dry starch basis, ds) before the starts of hydrolyses.

For the study of temperature effect on raw cassava starch hydrolysis, starch slurries were incubated at 30-40 °C with StargenTM002 at a concentration of 0.10% (v/w ds). The effect of pH on raw cassava starch hydrolysis was verified from 3.0−7.0. The starch slurries of pH 3.0−4.0 were prepared in sodium acetate buffer and of pH 5.0−7.0 were prepared in potassium phosphate buffer before the hydrolyses by StargenTM002 at a concentration of 0.10% (v/w ds).

4.2.2 Raw Cassava Starch Hydrolysi in 15-L Lysis Reactor

A 10-L of starch slurry, containing 20% (w/v) of raw cassava starch prepared in sodium acetate buffer at pH 4.0, was hydrolyzed by StargenTM002 at a concentration of 0.30% (v/w ds) in a 15-L lysis reactor at 40 °C and agitated at 220 rpm (equivalent to the power input , $\bar{\varepsilon}_T$ of 0.10 W/kg) for 72 h. During hydrolysis, the samples of 10 mL were withdrawn at every 12 h intervals for analysis. The pH value of each sample was adjusted to 1.5-1.6 with 2 M HCl solution to stop enzyme activities [14].

366 ***4.2.3 Raw Cassava Starch Pretreatments and Hydrolysis 15-L Lysis Reactor***
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368 The heat treatment of cassava starch at its below gelatinization temperature before being subjected
369 to enzyme hydrolysis can increase the degree of conversion (hydrolysis) of native starch to release free
370 glucose molecules [14]. In addition, [35] also reported that the combination of urea addition and
371 pre-heating treatment at a sub-gelatinization temperature greatly facilitated the hydrolysis by
372 StargenTM002. Therefore, in this study, a 10-L of starch slurry, containing 20% (w/v) of raw cassava
373 starch prepared in sodium acetate buffer at pH 4.0, was pretreated with heating at 60 °C in the 15-L lysis
374 reactor (in-house design and fabrication by our group, fit with 2 Ekato Intermig impellers of the
375 diameter, D of 0.115 m, (Germany) agitating at 220 rpm ($\bar{\varepsilon}_T$ = 0.10 W/kg) for 1 h, and with the additions of
376 (i) Distillase ASP at the concentrations of 0.10-0.30% (v/w ds) and (ii) urea at the concentrations of
377 1.0-3.0% (v/w ds). After pretreatment, the starch slurries were hydrolyzed by StargenTM002 at a
378 concentration of 0.30% (v/w ds) at 40 °C and stirring at the same speed of 220 rpm and $\bar{\varepsilon}_T$ = 0.10 W/kg for
379 72 h.
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381 ***4.2.4 Single-Step Ethanol Production Using Combination of Raw Cassava Starch Hydrolysis and***
382 ***Fermentation at Different Temperatures at the Laboratory Scale***
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384 In order to study the effect of temperatures on single-step ethanol production, the fermentations
385 were performed at different temperatures of 30, 40 °C, and without temperature control (with the initial
386 temperature of 40 °C). The pH values were not controlled with the initial value of 5.5 °C. They were
387 carried out using the 5-L fermenters (Biostat B, B. Braun Biotech Internationl, Germany) fit with 2
388 Rushtun turbine impellers of the diameter, D of 0.065 m, operating at the designed agitation speed of 200
389 rpm, equivalent to the power input, $\bar{\varepsilon}_T$ of 0.10 W/kg , for 72 h. The 5-L fermenters were contained with 4
390 L of fermentation media which were composed of 200 g/L (18% w/v dry solid, because of 10% moisture
391 content deduction) of pretreated raw cassava starch slurry plus 40 g/L (4% w/v) of sugarcane molasses
392 (containing 50% (w/v) sucrose, (to meet the total carbon source concentration of 20% (w/v)), and 0.1 g/L
393 $(NH_4)_2HPO_4$, 1.5 g/L of HK_2PO_4, 1.8 g/L of Na_2HPO_4, and 3.8 g/L of $MgSO_4.7H_2O$. After homogeneous
394 medium mixing, StargenTM002 was added at a concentration of 0.30% (v/w ds) and the inoculants of
395 re-hydrated *S. cerevisiae* actively dried yeast, Fali, were further inoculated into the fermenters at a final
396 concentration of 1.0 g/L (0.1% w/v). During fermentations, the samples of 10 mL were withdrawn at
397 every 12 h intervals for analyses.
398
399 ***4.2.5 Single-Step Ethanol Production Using Combination of Raw Cassava Starch Hydrolysis and***
400 ***Fermentation at the 200-L Pilot and the 3,000-L Industrial Scales***
401
402 (i) The pilot-scale ethanol fermentation was carefully conducted using the 200-L fermenter of 0.50 m
403 diameter, T and 1.00 m height, H (in-house design and fabrication by our group, fit with 2 Ekato Intermig

impellers of the diameter, D of 0.30 m, agitating at 125 rpm (equivalent to the power input, $\bar{\varepsilon}_T$ of 0.10 W/kg). The fermenter was contained with 150 L of fermentation medium^{P}.

(ii) The industrial ethanol fermentation was practically implemented using the 3,000-L fermenter of 1.25 m diameter, T and 2.50 m height, H (industrial design by our group and fabrication by Chorchiwat Industry Co., Ltd. (CCW)), fit with 2 Ekato Intermig impellers of the diameter, D of 0.85 m, agitating at 55 rpm (equivalent to the power input, $\bar{\varepsilon}_T$ of 0.10 W/kg). The fermenter was contained with 2,100 L of fermentation medium^{E}.

The fermentation media^{PandE} of both scales were definitely the same in compositions and concentrations. They were composed of 20% (w/v) of pretreated raw cassava starch slurry plus 4% (w/v) of sugarcane molasses (containing 50% (w/v) sucrose, and 0.1 g/L $(NH_4)_2HPO_4$, 1.5 g/L of HK_2PO_4, 1.8 g/L of Na_2HPO_4, and 3.8 g/L of $MgSO_4.7H_2O$. When mixing well and reached the design set point temperature of 34 °C and the initial pH of 5.5 (after 24 h, pH declined to 4.5 constant) the enzyme StargenTM002 was added in to the fermenters at a concentration of 0.30% (v/w ds) and further the inoculum of re-hydrated *S. cerevisiae*, Fali actively dried yeast was inoculated at a final concentration of 0.10%. The fermentations were performed at the temperature of 34 ±1 °C for 72 h at the design agitation speed of 110 rpm). During fermentations, the samples of 10 mL were withdrawn at every 12 h intervals for further analyses.

The studies of single-step ethanol production at the pilot-scale of 200-L and the industrial scale of 3,000-L fermenters were practically based on the principles of scale-up rules: (i) Geometric similarity in which all the dimensional lengths of the geometry among different fermenter scales were designed to maintain the same values. (ii) Condition similarity at which all the optimal conditions i.e. temperature, pH, substrate and enzyme concentrations were imitated from laboratory research works (iii) Operational similarity in which a selected key operation method was designed. For this work, the power input (energy dissipation per unit mass, $\bar{\varepsilon}_T$) was maintained similar (kept constant) with a value of $\bar{\varepsilon}_T = 0.10$ (W/kg) thus the impeller agitation speeds among different scales were calculated (see details below).

4.2.6 Analyses and Quantitative Methods

(1) Analytical Method using HPLC to Quantify the Concentrations of Glucose, Ethanol, and By-Products of Glycerol, Lactic acid, and Acetic acid

The High Performance Liquid Chromatography (HPLC) method was utilized to quantify the concentrations of glucose, ethanol, glycerol, lactic acid, and acetic acid in the samples by comparisons with the standards of those known concentration values. The HPLC (KNAUER Smartline, Berlin, Germany) with a refractive index (RI) detector (KNAUER Smartline 2300, Berlin, Germany) with the Eurokat H vertex column (KNAUER, Berlin, Germany) were used. An eluent of 0.01 N sulfuric acid at a

flow rate of 0.8 mL/min was utilized. The analyses were performed at 60 °C. The samples were 10-fold diluted, filtered through the 0.45 μm filter, and injected into the column with an amount of 20 μL.

(2) Total Sugar Analysis

Total sugars mean all the concentrations of carbon sources i.e., raw starch plus free residual glucose of which raw starch in the mixture of cell culture was completely hydrolyzed by acid to release all glucose molecules, and then glucoses from both sources in the same mixture were measured as the total sugar or total glucose. The total sugar, of culture broth during cell growth was analyzed by modified sulfuric acid hydrolysis method. The culture broth samples of 1 mL in micro-centrifuge each tube were centrifuged at 10,000 rpm for 10 min. The supernatants of 0.5 mL were transferred into each 20 mL test tube and then 2 mL of 2 N H_2SO_4 solution were added, mixed well, and capped. The test tubes with the mixture were boiled in a water bath at 95 °C for 30 min. Neutralizations were done with the addition of 4 N NaOH and re-centrifugation to precipitate the residues. Supernatants were measured glucose concentration as the total sugar by HPLC method.

(3) Scanning Electron Microscopy

The photographic characteristics of the pretreated and the hydrolyzed raw cassava starch grain was observed under the scanning electron microscope (SEM). The samples were mounted on the circular aluminum stubs with carbon tape, coated with gold, and examined and photographed in SEM (LEO, 1450VP, Germany) at an accelerating voltage of 10 kV.

(4) Kinetic Parameters' Calculations

(4.1) Starch Hydrolyses

The degree of conversion of raw starch to glucose was calculated from the release of glucose from raw cassava starch hydrolysis and those were obtained in the various experiments using the equation:

$$\text{Degree of conversion (\%)} = \frac{\text{Glucose release (g/L)}/1.11^{*}}{\text{Initial raw cassava starch (g/L)}} \times 100 \quad (1)$$

where 1.11 is the 1.0 g yield of glucose from 1.0 g of starch.

(4.2) Ethanol Productions

The 3 kinetic parameters of ethanol production were calculated using experimental data, i.e. cultivation time, t (h), concentration of ethanol, p (g/L), and concentration of glucose carbon substrate used, s (g/L). (i) Yield coefficient of ethanol product, $Y_{p/s}$ (g/g) is from $Y_{p/s} = \frac{\Delta p}{\Delta s}$, where Δp is the ethanol produced (g/L) and Δs is the substrates used (g/L), (ii) Productivity or production rate of ethanol, r_p (g/L/h) is from $r_p = \frac{dp}{dt}$. (iii) Efficiency of ethanol production, Ef (%) is from $Ef = \frac{Y_{p/s}}{Y'_{p/s}} x100$, where $Y_{p/s}$ is from the experiment and $Y'_{p/s}$ is from the theoretical yield coefficient or stoiciometry. The

theoretical yield of ethanol from starch is 0.567 g/g, calculated from $Y_{p/s} = \frac{0.511^{a} \times 1.11^{b}}{1.0}$, Where [a] is the theoretical yield (g) of ethanol from 1.0 g glucose and [b] is the yield of glucose (g) from 1.0 g starch.

***(5) Calculation* of *the Designed Impeller Speed*s**

For fluid dynamics in the stirred tank bioreactor [36], the power, P (W) is calculated from $P = nPo\rho N^3 D^5$, where n is the number of impellers of 2 for all the 5-L fermenter, 15-L lysis reactor, 200 pilot-scale fermenter and 3,000-L industrial fermenter. The Po is the power number of 5 (no unit or dimensionless), for one Rushton turbine impeller of the 5-L fermenter. The Po is 0.33 for one Ekato Intermig (high efficiency) impeller of every scale, i.e., 15-L lysis reactor, 200-L and 3,000-L fermenters. ρ is the fluid densities (kg/m^3) of lysis reactor or fermenters. N is the impeller speeds (rps), and D is the impeller diameters of 0.05 m for 5 L-fermenter, 0.115 m for 15-L lysis reactor, 0.30 m for 200 L and 0.85 m for 3,000-L fermenters.

The power input or energy dissipation per unit mass, $\bar{\varepsilon}_T$ (W/kg) is calculated from

$$\bar{\varepsilon}_T \text{ or } \frac{P}{\rho V} = \frac{nPo_g \rho N^3 D^5}{\rho V} \quad (2)$$

where V is the fluid volume (m^3). Thus, the design impeller speeds, N, of the power inputs of 0.10 (W/kg) are calculated from

$$N = \left(\frac{\bar{\varepsilon}_T V}{nPo\rho D^5}\right)^{1/3} \quad (3)$$

These both kinetic and fluid dynamic parameters from the laboratory and pilot-scale experiments are very crucial for the scale-up of further fermentations at the industrial scales. That is the foreseen reason why we design the impeller speeds based on the power input rather than just the speed in rpm (Table 6).

Table 6. Lysis reactor and fermenter designs, and scale-up valves for this research work; they were designed by maintaining geometric similarity and power input, $\bar{\varepsilon}_T$ among different fermenter scales constant with the value of 0.10 W/kg

Reactor Types and Total Vol. (L)	Working Vol. (L)	Dimensions H/T (m/m) Height/Tank ø	Impeller Types	No.	Impeller Diameters, D (m)	Speeds (rpm)	Power Input, $\bar{\varepsilon}_T$ (W/kg)
15-L Lysis Reactor	10	0.40 / 0.20	Ekato Intermig	2	0.115	220	0.10
5-L Fermenter	4	0.35 / 0.16	Rushton Turbine	2	0.065	200	0.10
200-L Fermenter	150	1.00 / 0.50	Ekato Intermig	2	0.300	125	0.10
3,000-L Fermenter	2,100	3.00 / 1.50	Ekato Intermig	2	0.850	55	0.10

(6) Statistical Analysis

The statistical analyses of each data set from each experiment were done with the One−Way Analysis of Variance (ANOVA). The differences of the treatment mean values from 3 replications (each experiment was done in 3 replicates) were compared with the Tukey's Range Test method at p-value ≤ 0.05 using software Minitab version 17. The standard error ± SE were shown together with the mean values of figure numbers and shown as the error bars in the graphs. If the error bars do not emerge, it is assumed that they are smaller than the size of the symbols.

Abbreviations

CBP: consolidated bioprocessing; $Y_{p/s}$: product yield coefficient; r_P: $Y'_{p/s}$: theoretical yield coefficient; r_P: productivity or production rate; Ef: efficiency; SSF: simultaneous saccharification and fermentation; w/w: weight by weight; v/v: volume by volume; v/w ds: volume by weight of dry solid; g/L/h : gram per liter per hour; L: liter; m: meter; H^+-ATP: hydrogen ion-adenosine triphosphate; g/g: gram per gram; g/L: gram per liter; $\bar{\varepsilon}_T$: power input or energy dissipation per unit mass; W/kg: Watts per kilogram; GAU: glucoamylase unit; (GAU), rmp: revolutions per minute; rps: revolutions per second; M HCl: molar of hydrochloric acid; $(NH_4)_2HPO_4$: diammonium hydrogen phosphate; HK_2PO_4: hydrogen dipotassium phosphate; Na_2HPO_4: disodium hydrogen phosphate; $MgSO_4.7H_2O$: magnesium sulphate; N H_2SO_4: normal sulfuric acid; N NaOH normal sodium hydroxide; H: height; T: tank diameter; D: impeller diameter; CCW: Chorchiwat Industry Co., Ltd.; HPLC: high performance liquid; μm: micro meter; μL: micro liter; SEM: scanning electron microscope; p: concentration of ethanol product; t: time; s: carbon (glucose or starch) substrate; Δp: ethanol produced; Δs : substrates used; d: differential; P: power; n: number of impellers; Po: impeller power number; N: impeller speed; ρ: fluid density; V: volume; Ø: diameter; ANOVA: analysis of variance; SE: standard error.

Acknowledgements

This work was supported by the Biological Science Graduate Program and Department of Biology, Faculty of Science, Burapha University, Chonburi, Thailand. The authors would like to sincerely thank Chorchiwat Industry Co., Ltd. (CCW) who supported necessary facilities, raw materials, some experiments, and analytical instruments for this study. Also grateful thanks to the CCW research and development office and BS6201 team for their helps.

Author Contributions

Conceptualization, M.K. and K.M.; methodology, investigation, analysis and writing—original draft preparation, JS. and K.R. ; resources, validation, visualization, and data curation, S.C.; writing—review and editing, validation, visualization, supervision, project administration and funding acquisition.

Funding

A research grant was from the "Office of the Higher Education Commission". This work was also supported by the Biochemical Engineering Pilot Plant, Biological Science Graduate Program, and Department of Biology, Faculty of Science, Burapha University, Chon Buri, Thailand.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests. The funder had no role in the design of this study, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the experimental results.

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Received: xxxxx **Accepted**: xxxxxx

Published online: xxxxx

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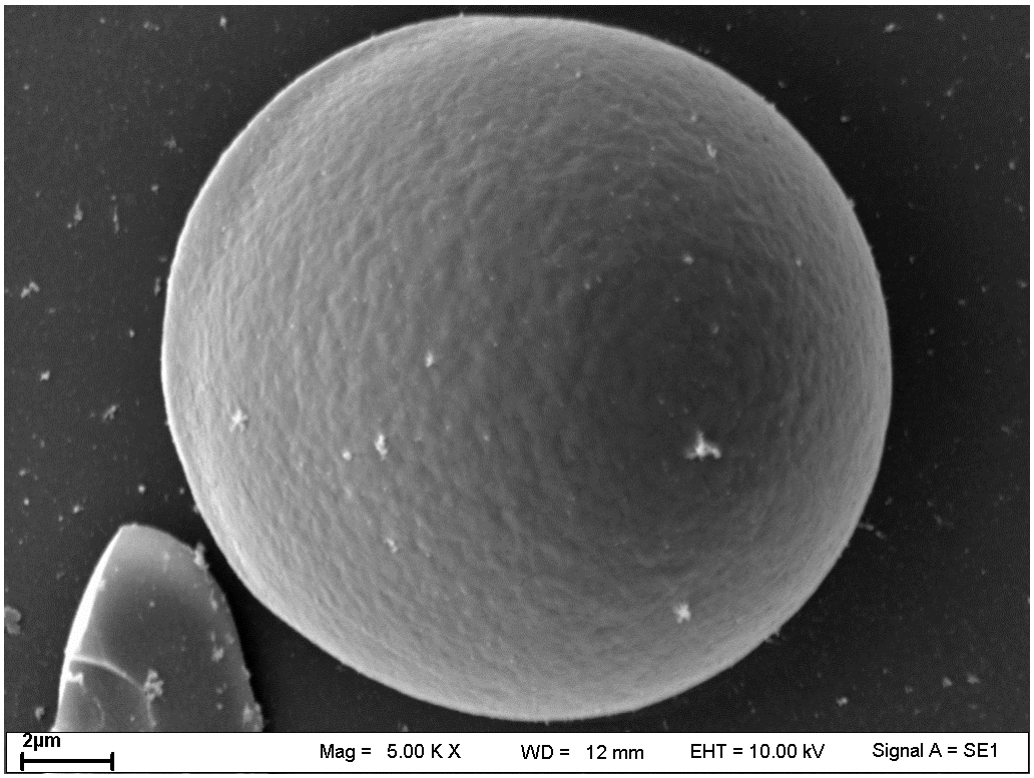
649 (a)



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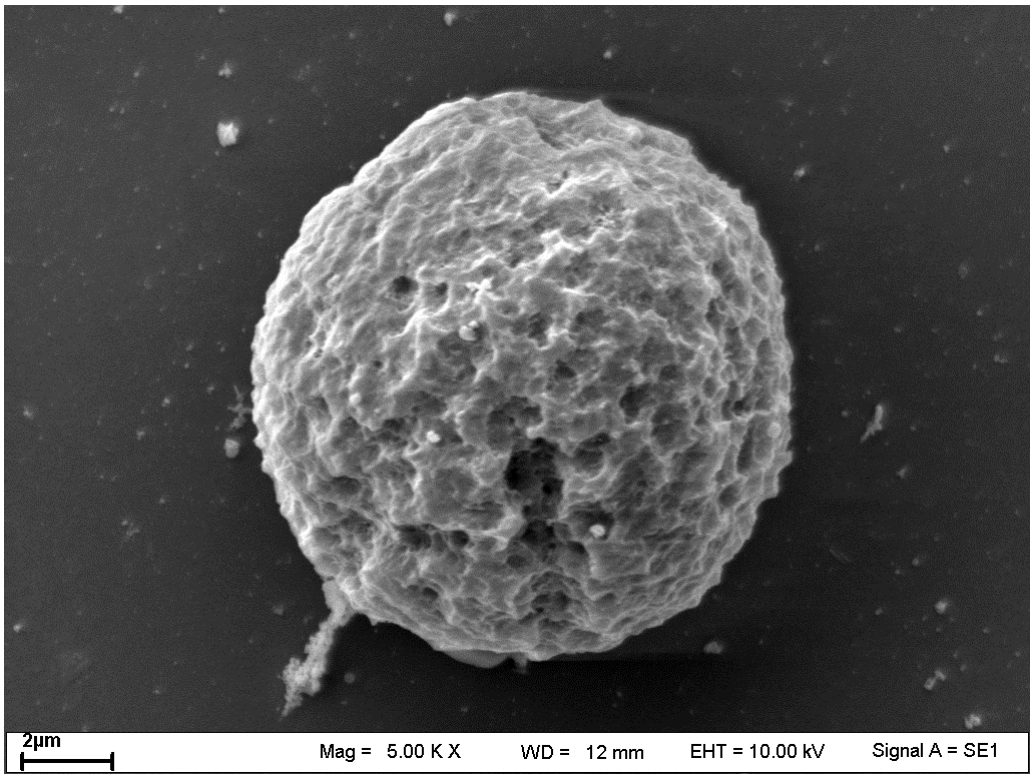

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654 **Figure 1.** Scanning electron micrographs of (a) pretreated raw cassava starch granule at sub-gelatinization
655 temperature of 60 °C with Distillase ASP for 1 h and (b) hydrolyzed raw cassava starch granule by
656 Stargen™002 for 48 h.

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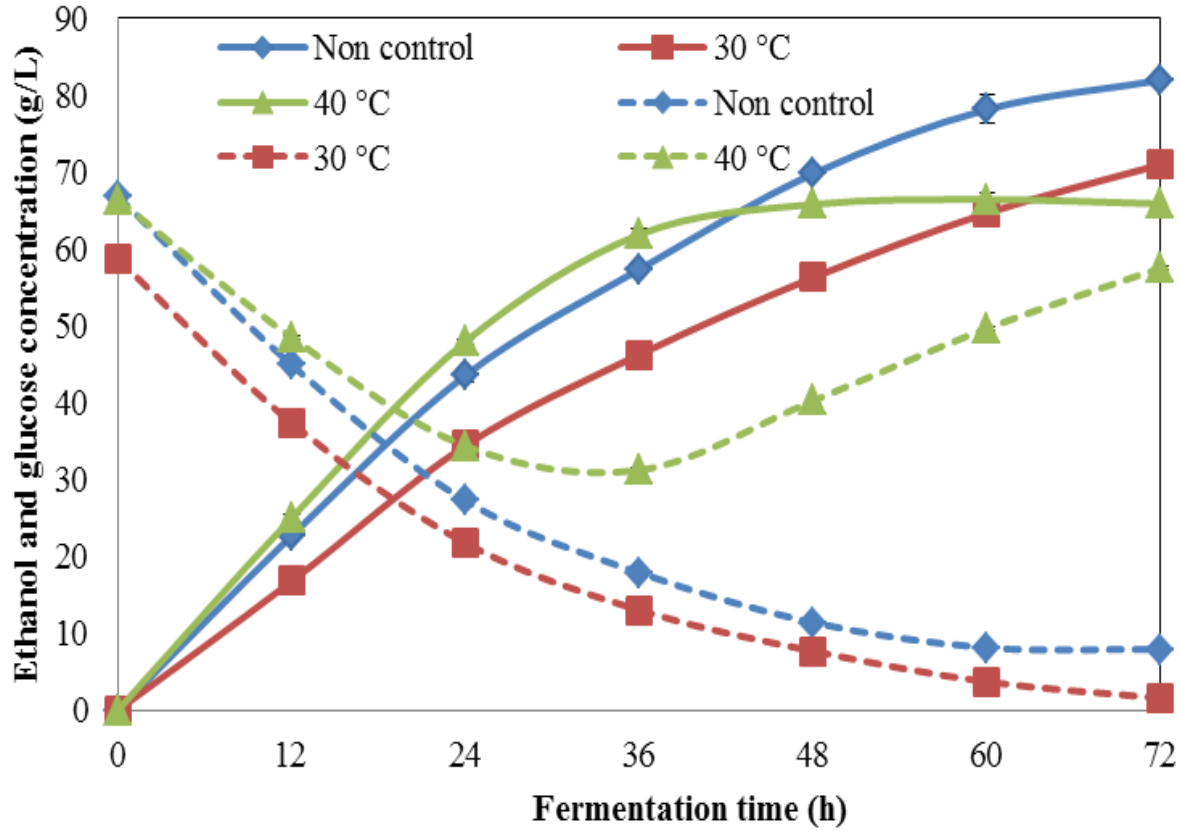

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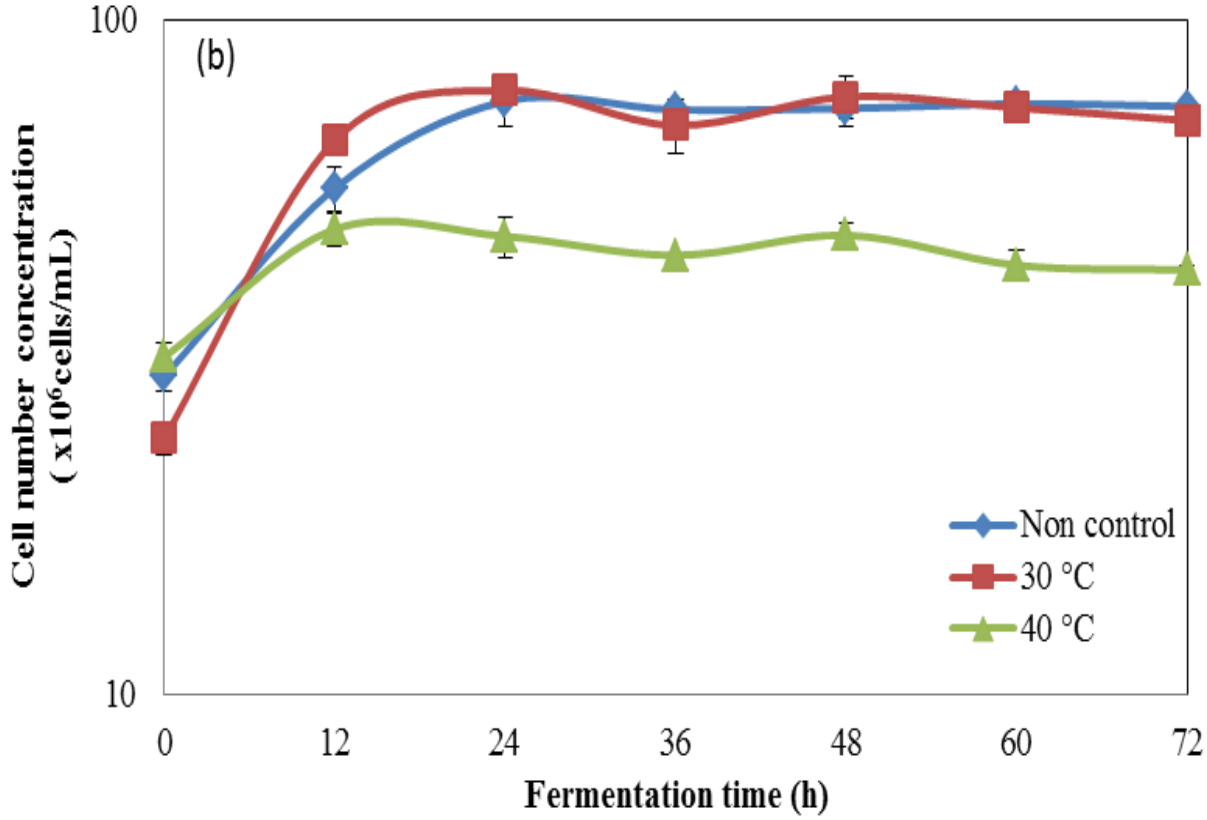

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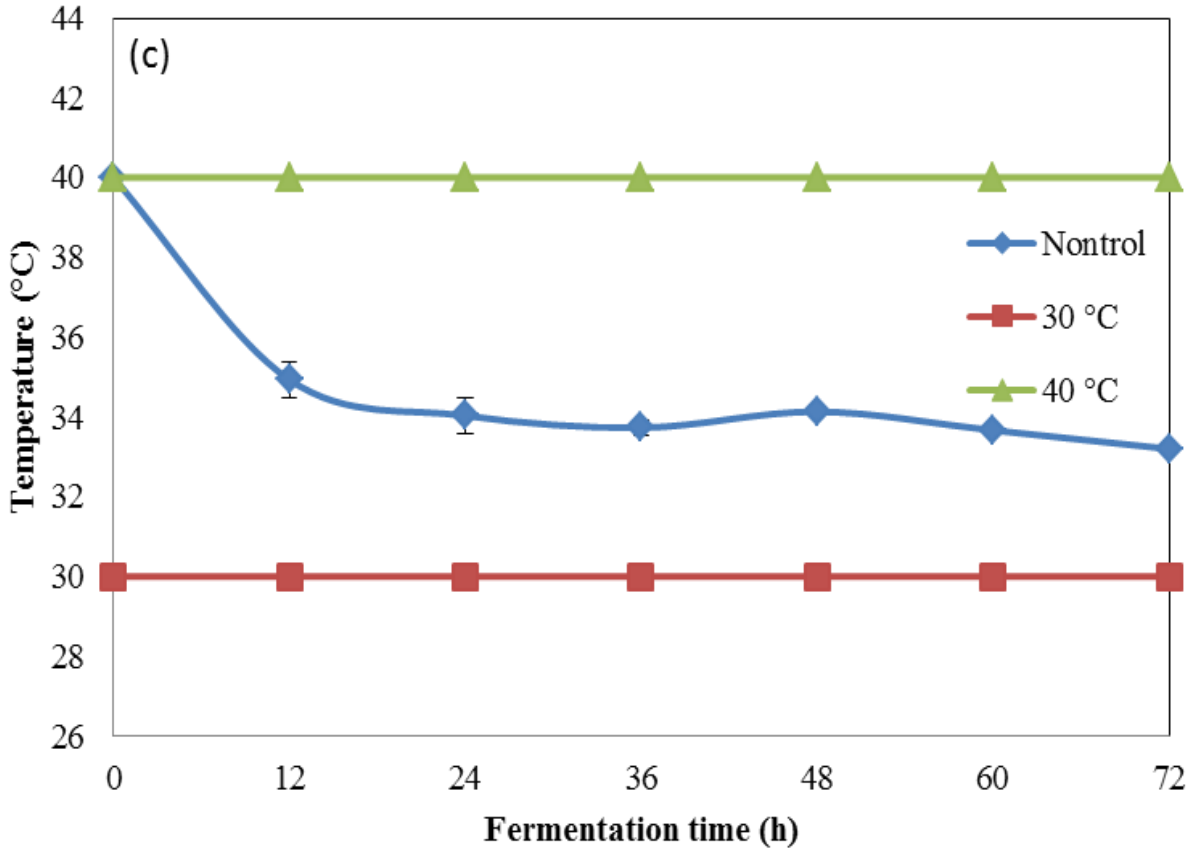
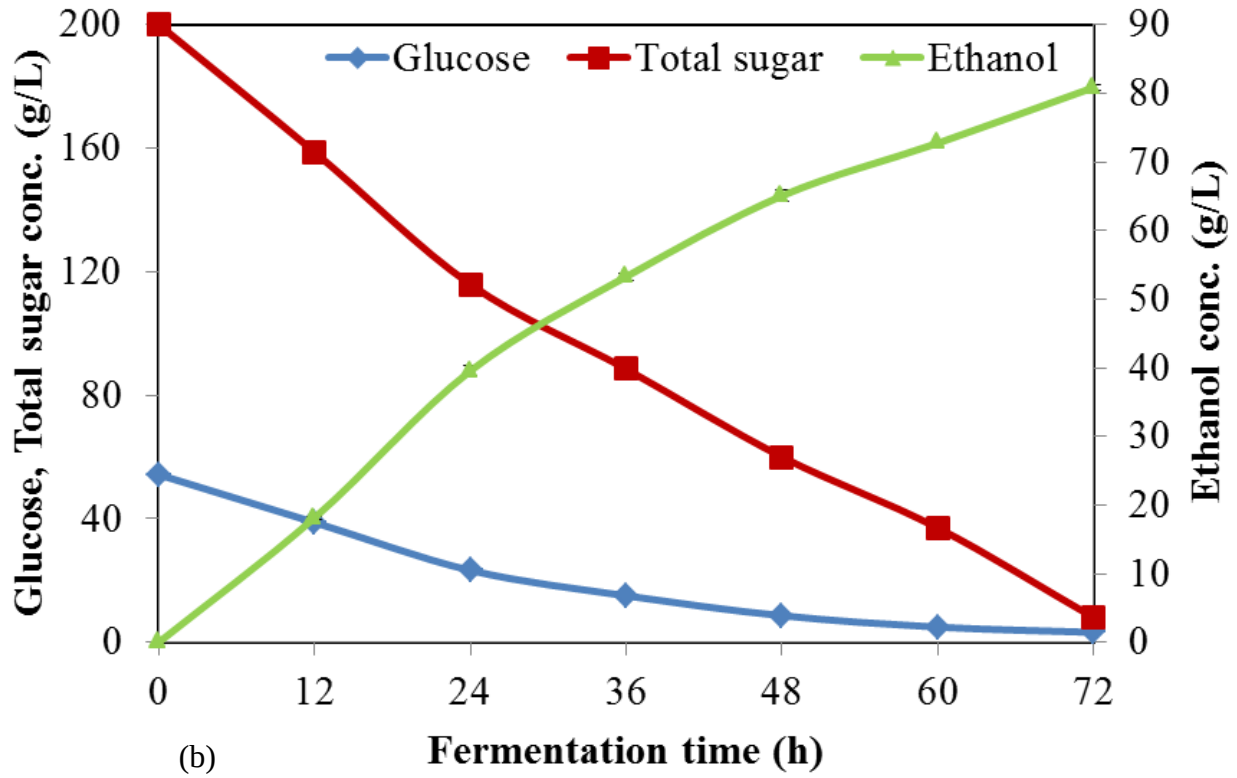


Figure 2. Ethanol production and sugar utilizations (a), cell number density concentration of *S. cerevisiae* (b), and temperature profiles (c) during single-step ethanol fermentation at different temperatures in 5-L fermenters.

(a)



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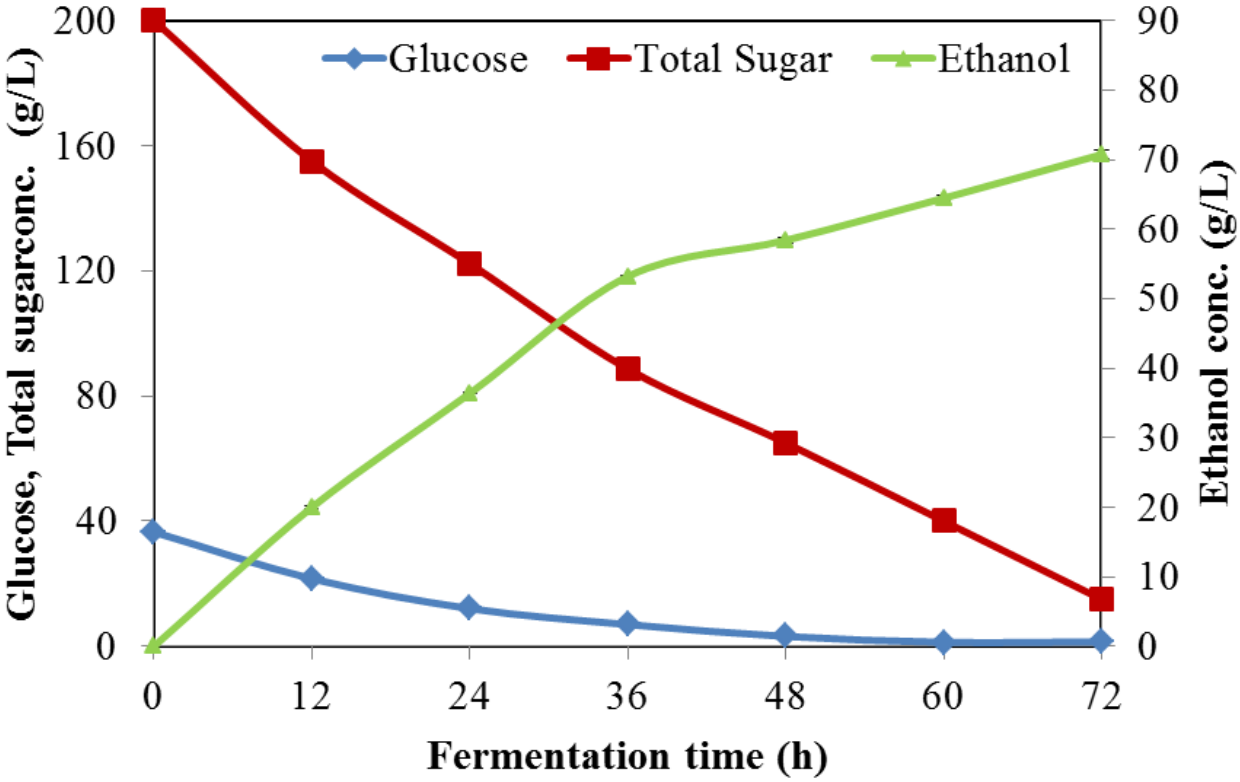

Figure 3. Ethanol productions and substrate utilizations by single-step ethanol fermentations in (a) 200-L pilot-scale fermenter and (b) 3,000-L industrial fermenter

690 **Table 1.** The release of glucose from raw cassava starch, productivity, and degree of conversion of raw cassava
691 starch to glucose by Stargen™002 at various enzyme dosages, temperatures, and initial pH values
692

Main factors	Glucose concentration (g/L)	Productivity, r_p (g/L/h)	Degree of conversion to glucose (% w/w)
Stargen™002 (% w/w)			
0.1	37.45 ± 5.98[a]	0.99 ± 0.27[a]	17.02 ± 2.72[a]
0.2	56.56 ± 1.36[b]	1.18 ± 0.03[b]	25.71 ± 0.62[b]
0.3	73.78 ± 4.18[c]	1.54 ± 0.09[c]	33.54 ± 1.90[c]
0.4	78.70 ± 1.41[c]	1.64 ± 0.03[c]	35.77 ± 0.64[c]
Temperature (°C)			
30	30.07 ± 0.39[a]	0.63 ± 0.01[a]	13.67 ± 0.18[a]
35	59.50 ± 5.31[b]	1.24 ± 0.11[b]	27.05 ± 2.41[b]
40	133.27 ± 7.83[c]	2.78 ± 0.16[c]	60.58 ± 3.56[c]
Initial pH			
3.0	114.39 ± 1.24[a]	2.38 ± 0.03[a]	52.00 ± 0.56[a]
4.0	96.39 ± 1.03[b]	2.01 ± 0.02[b]	43.82 ± 0.47[b]
5.0	56.26 ± 1.17[c]	1.17 ± 0.02[c]	25.57 ± 0.53[c]
6.0	33.10 ± 1.14[d]	0.69 ± 0.02[d]	15.04 ± 0.52[d]
7.0	16.59 ± 0.36[e]	0.35 ± 0.01[e]	7.70 ± 0.16[e]

693 Statistic comparisons of those mean values within their own columns (among main factors) at *p*-values ≤
694 0.05 show different characters, a, b, c, d, and e, which mean statically significant differences.
695
696

697 **Table 2.** Glucose formation and degree of conversion of starch to glucose from pretreatment of raw cassava
698 starch and further hydrolysis by Stargen™002 at the optimum conditions for 72 h
699

Starch slurry pretreatment (1 h)			Hydrolysis by Stargen™002 (40 °C, 72 h)		
Temperature (°C)	Distillase ASP (% w/w)	Urea (% w/w)	Glucose concentration (g/L)	Productivity, r_p (g/L/h)	Degree of conversion to glucose (% w/w)
None pretreated			150.83 ±1.58[a]	2.09 ±0.02[a]	68.56 ±0.72[a]
60	-	-	159.90 ±2.85[b]	2.22 ±0.04[b]	72.68 ±1.30[b]
60	0.1	-	176.41 ±1.52[c]	2.45 ±0.02[c]	80.19 ±0.69[c]
60	0.2	-	173.81 ±2.28[c]	2.41 ±0.07[c]	79.00 ±1.20[c]
60	0.3	-	175.93 ±1.99[c]	2.44 ±0.02[c]	79.97 ±1.81[c]
60	0.1	1.0	167.81 ±1.51[d]	2.33 ±0.02[d]	76.28 ±0.68[d]
60	0.1	2.0	171.55 ±8.09[d]	2.38 ±0.11[d]	77.93 ±3.68[d]
60	0.1	3.0	166.47 ±1.15[d]	2.31 ±0.02[d]	75.67 ±0.52[d]

700 Statistic comparisons of those mean values within their own columns (among slurry pretreatments) at
701 *p*-values ≤ 0.05 show different characters, a, b, c, and d, which mean statically significant differences.
702
703
704

Table 3. Comparison of the kinetic parameters of the single-step ethanol productions using combination of raw cassava starch hydrolysis and fermentation at different scales of 5-L, 200-L, and 3,000-L fermentations by *S. cerevisiae* for 72 h

Fermentation	Ethanol (g/L)	Ethanol % (v/v)	$Y_{p/s}$, Yield coefficient (g/g)	Productivity, r_P (g/L/h)	Efficiency (%)
5-L fermenter	81.86 ± 1.88[a]	10.43	0.41 ± 0.01[a]	1.14 ± 0.03[a]	71.44 ± 1.66[a]
200-L fermenter	80.85 ± 0.45[a]	10.23	0.41 ± 0.00[a]	1.12 ± 0.01[a]	72.47 ± 0.39[a]
3,000-L fermenter	70.74 ± 0.56[b]	9.01	0.34 ± 0.00[b]	0.98 ± 0.01[b]	59.82 .± 0.47[b]

Statistic comparisons of those mean values within their own columns (among fermentation scales) at p-values ≤ 0.05 show different characters, a, b, c, and d, which mean statically significant differences.

Table 4. The concentrations of by-products of glycerol, lactic acid, and acetic acid from single-step ethanol productions using combination of raw cassava starch hydrolysis and fermentation at different scales of 5-L, 200-L, and 3,000-L fermentation by *S. cerevisiae* for 72 h.

Fermentations	Glycerol (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
5-L fermenter	11.39 ± 0.11	0.46 ± 0.00	0.00 ± 0.00
200-L fermenter	11.02 ± 0.03	0.51 ± 0.01	0.23 ± 0.01
3,000-L fermenter	9.39 ± 0.07	0.52 ± 0.01	0.59 ± 0.00

No statistic comparisons of those mean values within their own columns (among fermentation scales) because those values are very low and less than those of the values to inhibit yeast cell growth and affect ethanol yield.