Production of high-concentration bioethanol from cassava stem by repeated hydrolysis and intermittent yeast inoculation

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ABSTRACT

Methods for obtaining high-concentration (ca. 40 g/L) ethanol from cassava stem (CS) were explored by investigating the effects of acid hydrolysis conditions, hydrolysate concentration, and intermittent inoculation of yeast on ethanol fermentation. Two-step acid hydrolysis demonstrated that a reduction in autoclaving temperature from 121 °C to 111 °C remarkably lowered the furfural concentration in the hydrolysate, with only ± 10% differences in the glucose and xylose concentrations. For the concentration of sugars in the CS hydrolysate, the acid hydrolysate prepared with CS concentration of 200 g/L was used instead of distilled water for the second step of acid hydrolysis using new CS biomass (i.e., two-times acid hydrolysis). This produced a concentrated hydrolysate containing 95 g/L of glucose and 0.95 g/L furfural. *Saccharomyces cerevisiae* strain IAM 4178 was inoculated into the concentrated CS hydrolysate after verifying its furfural tolerance. Furfural was rapidly degraded and glucose was converted to ethanol during the startup period, whereas cell density decreased to approximately 10% of the initial value and ethanol production ceased by 72 h. IAM 4178 was then re-inoculated intermittently to maintain the cell density at 10^8 cells/mL, which eventually resulted in the complete uptake of glucose and the production of high concentrations of ethanol, up to 37.5 g/L.

1. Introduction

Lignocellulose-rich agricultural waste is a solid waste that is abundant, organic in nature, and found around the world. The effective treatment or utilization of this agricultural waste has been explored. Bioethanol fermentation of agricultural waste has recently attracted attention because it does not compete with food supply (Shields and Boopathy, 2011), unlike conventional bioethanol fermentation processes using crops such as sugarcane or corn. Cassava stem (CS) is an abundant lignocellulosic agricultural waste generated mainly in Africa, South America, and Southeast Asia. As much as 80–90% of CSs are not utilized and are abandoned or simply burned. However, being composed of 35–42% cellulose and 15–24% hemicellulose (Han et al., 2011; Klinpratoom et al., 2015), CS is a potential biomass for the generation of fermentable sugars for bioethanol production.

In the ethanol fermentation of lignocellulose, pretreatment is necessary for the destruction of rigid lignocellulose structures and for the hydrolysis of polysaccharides (i.e. cellulose and hemicellulose) into monosaccharides. The increase of monosaccharide concentration in the hydrolysate is very important for obtaining high concentrations of ethanol (Yadav et al., 2011). Acid hydrolysis with sulfuric acid, is the most common method to disrupt

lignocellulose. A number of studies have applied acid hydrolysis for the enhancement of fermentable sugar yield (Fernandes et al., 2018; Li et al., 2018; Sun et al., 2011). The concentration of ethanol from acid hydrolysate of lignocellulose biomass is typically 10–35 g/L (Taherzadeh and Karimi, 2007). Few studies have investigated the ethanol productivity of CS using dilute sulfuric acid and cellulase (Han et al., 2011), $NaClO_2$ + NaOH and dilute sulfuric acid (Klinpratoom et al., 2015), and heat and cellulase (Nanssou et al., 2016). Han et al. (2011) applied acid hydrolysis (up to 0.14 M H_2SO_4) followed by enzymatic saccharification for the pretreatment of bioethanol fermentation of CS, which resulted in an ethanol yield of 7.55 g/L. Klinpratoom et al. (2015) also conducted two-stage chemical pretreatment ($NaClO_2$ followed by NaOH) and obtained an ethanol yield of 13.52 g/L. However, the concentrations of the obtained ethanol are considered low, because high ethanol concentrations of approximately 40 g/L or above are often recommended (Akinosho et al., 2014; Lu et al., 2010) owing to the requirement of compensation for the high energy input at the distillation step. To date, only a few studies have achieved 40 g/L ethanol in bioethanol fermentation of agricultural residue by using genetically modified organisms (GMO) such as *Escherichia coli* (Moniruzzaman and Ingram, 1998) or *Saccharomyces cerevisiae* (Lee et al., 2017) for producing ethanol

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not only from glucose but also from xylose. Accordingly, an operational method without applying GMO for obtaining high-concentration ethanol from CS should be developed.

Acid recycling (or two-times acid hydrolysis), which uses the primary acid hydrolysate as an acid hydrolysis solution for a secondary hydrolysis of new biomass (Cheng et al., 2008) seems feasible to obtain concentrated fermentable sugar (both C6 and C5 sugars) in the resulting hydrolysate. Indeed, Cheng et al. (2008) reported the increase of reducing sugar concentration from 28 to 63.5 g/L after two-times acid hydrolysis of sugarcane bagasse and obtained 19 g/L ethanol. Conversely, acid hydrolysis produces fermentation inhibitors such as furfural and phenolic compounds, which can lead to low ethanol productivity (Palmqvist and Hahn-Hägerdal, 2000a). Thus, the removal of such inhibitors (i.e. detoxification) by activated carbon adsorption (Gong et al., 1993), or overliming to induce precipitation of inhibitors under alkaline conditions (Palmqvist and Hahn-Hägerdal, 2000a) is required to make high concentration ethanol fermentations achievable. However, loss of fermentable sugars can occur during detoxification by non-selective adsorption or precipitation (Moniruzzaman and Ingram, 1998; Schirmer-Michel et al., 2008). Accordingly, a process devoid of such detoxification should be developed to obtain high concentration ethanol production.

During the fermentation of concentrated acid hydrolysate without detoxification, inhibitors may kill yeast. In fact, it is reported that the growth of yeast can be inhibited by furfural or phenolics (Palmqvist and Hahn-Hägerdal, 2000b). On the other hand, high glucose consumption rate was maintained when the cell density was increased from 0.55 g/L to 2.5 g/L and from 2.5 g/L to 9.0 g/L, even under high furfural concentration of up to 5 g/L (Navarro, 1994), indicating that high cell density could improve ethanol productivity from inhibitor-containing hydrolysate. Therefore, intermittent inoculation of yeast might maintain high cell densities throughout the operational period and could produce high-concentration ethanol from concentrated (both sugars and inhibitors) hydrolysate. To our knowledge, no study has yet examined the effects of intermittent inoculation on the ethanol fermentation process.

Therefore, in order to obtain approximately 40 g/L ethanol from CS, the effect of hydrolysate concentration (two-time hydrolysis using recycled hydrolysate) and intermittent yeast inoculation on the ethanol fermentation of CS hydrolysate was examined in the present study. For the yeast, *Saccharomyces cerevisiae* IAM4178 was used as a commonly applied yeast for ethanol production from glucose. In addition, *Blastobotrys (Arxula) adeninivorans* XE1was also utilized in the present study, since our preliminary experiment revealed that this strain produces ethanol from both glucose and xylose.

2. Materials and methods

2.1. Raw materials and yeast strains

CS was obtained from Trang Bom region, Dong Nai prefecture, Vietnam. The moisture content of the air-dried CS was 6.4%, and the glucan and xylan content was 39.5 and 16.6% of dry solid (ds), respectively. The air-dried CS was shredded by a grinder (Verder Scientific Co., Ltd., ZM100) with the mesh screen (aperture 0.5 mm) and stored in a desiccator until use. Yeast strains *Saccharomyces cerevisiae* IAM 4178 and *Blastobotrys (Arxula) adeninivorans* XE1 were used for the fermentation of CS hydrolysate. These yeasts were maintained at 4 °C on Yeast malt (YM) agar medium (g L⁻¹): glucose 10.0, polypeptone 5.0, yeast extract 3.0, malt extract 3.0, agar 20.0 (pH 6.2). *S. cerevisiae* IAM 4178 and *B. adeninivorans* XE1 were precultured in YM liquid medium at 30 °C for 48 h. The preliminary fermentation experiments using 10 g/L glucose and/or 5 g/L xylose were conducted to examine the ethanol production capacity of each yeast strains. The fermentation condition was described in section 2.5.

2.2. Acid hydrolysis

The process flow diagram of the present study was summarized in Fig.

S1. Two-step acid hydrolysis using concentrated and diluted sulfuric acid was conducted in order to perform the saccharification of cellulose and hemicellulose (Sluiter et al., 2011). For the first step, ground CS and 72 wt% sulfuric acid were mixed at a weight ratio of 1:1.25 in a 1 L glass media bottle. The mixture was maintained at 30 °C for 1 h and stirred every 15 min to ensure uniformity. For the second step, a different volume of distilled water was added to change the CS concentration to 20 g/L (=CS20), 100 g/L (=CS100) and 200 g/L (=CS200), (the sulfuric acid concentration becomes 1.8, 8.6 and 16.4%, respectively), in order to investigate the optimum CS concentration. Thereafter, the mixture of CS and diluted acid was autoclaved at 121 or 111 °C for 1 h, in order to examine the effect of heating temperature on the sugar yield and the ethanol productivity. The mixture was kept at room temperature for 1 h, and calcium carbonate was added to adjust the pH to approximately 5.5. After neutralization, the mixture was centrifuged at 3980 × g for 5 min and the supernatant was filtered through a 0.45 μm membrane filter to separate the residual solids, and the filtrate was used as "acid hydrolysate". Before fermentation, 5 g/L of peptone and 3 g/L of yeast extract were supplemented to the CS hydrolysate.

2.3. Detoxification of CS hydrolysate

The effect of detoxification on ethanol production was investigated by using overliming and activated carbon. First, CS200 was prepared at 111 °C for 1 h. The hydrolysate was kept at room temperature for 1 h and calcium carbonate was added to adjust the pH to 11–12. This mixture became a slurry due to the production of gypsum. The mixture was centrifuged at 3980 × g for 5 min, the supernatant was separated, and sulfuric acid was added to adjust the pH to 5.5. The supernatant was filtered through a 0.45 μm membrane filter. Subsequently, granulated activated carbon was added to the filtrate at 10% w/v and stirred using magnetic stirrer for 1 h, and filtered through 0.45 μm membrane filter to remove activated carbon. Before fermentation, 5 g/L of peptone and 3 g/L of yeast extract were added to the CS hydrolysate.

2.4. Concentration of CS hydrolysate by two-times acid hydrolysis

In order to increase the concentration of fermentable sugars to the levels required for producing high-concentration ethanol by two-times acid hydrolysis, a first, concentrated hydrolysate prepared as described in section 2.2 was diluted with a volume of filtrate of a second concentrated hydrolysate equivalent to that of distilled water used for preparation of CS200 in section 2.2. The mixture of CS and hydrolysate was autoclaved at 111 °C for 1 h. The pH adjustment, centrifugation, filtration, and nutrient supplementation were performed in the same manner as indicated above.

2.5. Fermentation of CS hydrolysate

Ethanol fermentation of CS hydrolysate, pretreated at different

Table 1
Experimental conditions.

Run	Substrate conc. (g/L)	Temperature (°C)	Detoxification	Inoculum
1	20	121	–	IAM4178
2	20	121	–	XE1
3	100	121	–	IAM4178
4	100	111	–	IAM4178
5	100	111	–	XE1
6	200	111	–	IAM4178
7	200	111	–	XE1
8	200	111	Overliming + activated carbon	XE1
9	400[a]	111	–	IAM4178[b]

[a] Concentration by two-time hydrolysis.
[b] Intermittent inoculation.

hydrolysis temperatures and CS concentrations, was conducted using strains IAM 4178 and XE1. The operational runs (Run 1–9) are summarized in Table 1. For inoculation of yeast, 50 mL of the preculture medium of IAM 4178 or XE1 was firstly centrifuged at 3980 × g for 5 min. The supernatant was discarded and a wash solution (5 g/L peptone and 3 g/L yeast extract) was added to wash the yeast pellet. The mixture was centrifuged again and the supernatant was discarded. The obtained yeast pellet was inoculated into 50 mL CS hydrolysate of each run, and the fermentation was conducted at 30 °C, 120 rpm. All runs except Run 4, 8 and 9 were operated in duplicate, and reproducibility of the obtained data was confirmed.

2.6. Physicochemical and microbial analyses

Glucose, xylose, xylitol, ethanol, and furfural in the liquid culture were measured with an HPLC system, a Shodex Sugar SH1011 column, and a refractive index detector. The mobile phase was 5 mM sulfuric acid, the flow rate was 0.6 mL/min, and the temperature in the column oven was 60 °C. Theoretically, 0.51 g of ethanol is produced from 1 g of glucose, according to the formula of ethanol production from glucose. In the present study, ethanol conversion efficiency of glucose was calculated for all experiments. Cell density was measured by the dilution plating method using YM agar plates. Incubation was conducted at 30 °C, for 2 days. Phenolic compounds were measured by the Folin-Ciocalteu method, referred from Wei et al. (2013).

3. Results and discussion

3.1. Characteristics of ethanol production using the two yeasts

The time course of ethanol fermentation by strains IAM 4178 and XE1 on CS20 hydrolyzed at 121 °C (Runs 1 and 2) is shown in Fig. 1(a and b). Approximately 0.08 g/L of furfural was produced during acid hydrolysis, which was rapidly degraded within 6 h during the fermentation by both strains. IAM 4178 completely consumed glucose within 12 h of fermentation. Accordingly, the ethanol concentration peaked at 12 h. On the other hand, xylose was slowly consumed during the early fermentation period, but the majority of xylose remained in the hydrolysate even after 36 h. In our preliminary fermentation experiment using synthetic medium (glucose and/or xylose), we confirmed that IAM 4178 marginally degraded xylose neither in only xylose nor in the mixture of glucose and xylose (Fig. S2). It is known that *S. cerevisiae* consumes glucose but cannot consume xylose (Sarkar et al., 2012). In the present study, the contribution of xylose to ethanol production was considered to be negligible because the glucose-based ethanol conversion efficiency of CS20 was 0.422 g-ethanol/g-glucose, which was similar to that of the glucose + xylose of the synthetic medium in preliminary experiments.

Conversely, fermentation characteristics of CS20 by XE1 were different to those of IAM 4178. XE1 degraded glucose in approximately 18 h, indicating slower glucose uptake as compared with that of IAM 4178. The uptake of xylose started from 12 h and was completed by 30 h. Furthermore, a small amount of xylitol was produced as a result of
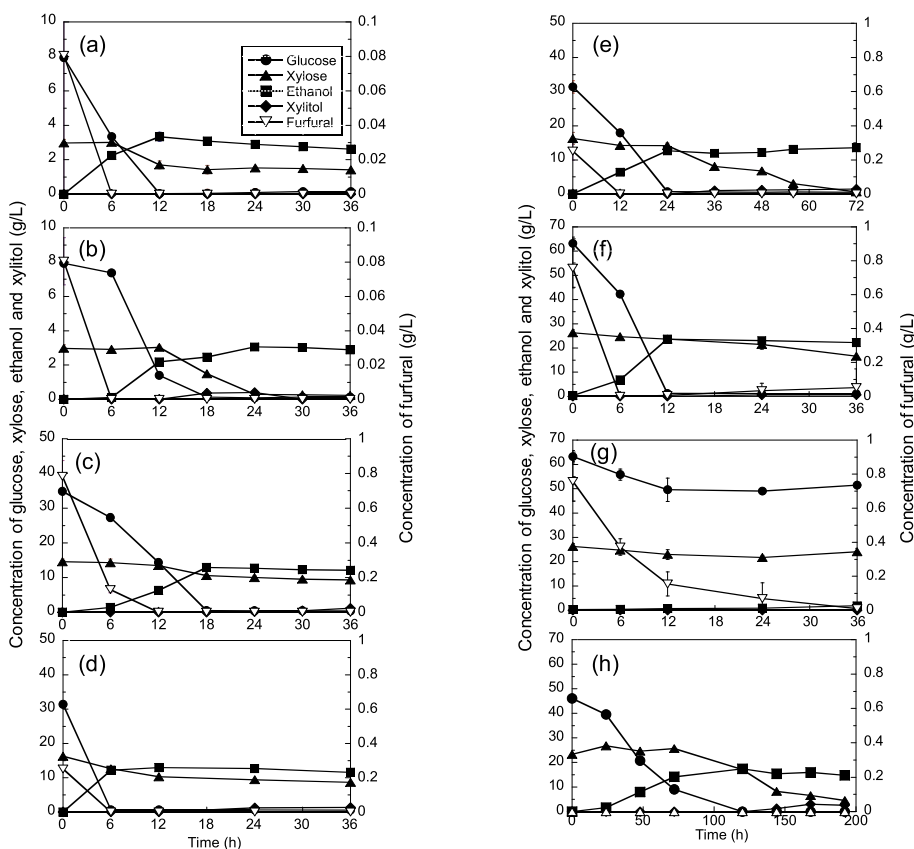
Fig. 1. Time course of sugars, furfural and ethanol concentrations during fermentation of cassava stem hydrolyzed at different acid hydrolysis condition by strain IAM 4178 and XE1. (a) Run 1: CS 20 g/L, acid hydrolysis 121 °C, IAM 4178, (b) Run 2: CS 20 g/L, acid hydrolysis 121 °C, XE1, (c) Run 3: CS 100 g/L, acid hydrolysis 121 °C, IAM 4178, (d) Run 4: CS 100 g/L, acid hydrolysis 111 °C, IAM 4178, (e) Run 5: CS 100 g/L, acid hydrolysis 111 °C, XE1, (f) Run 6: CS 200 g/L, acid hydrolysis 111 °C, IAM 4178, (g) Run 7: CS 200 g/L, acid hydrolysis 111 °C, XE1, (h) Run 8: CS 200 g/L, acid hydrolysis 111 °C, detoxification by overliming and activated carbon, XE1.

xylose degradation. Our preliminary experiments confirmed the uptake of xylose and the production of ethanol and xylitol during the fermentation of xylose by XE1 (Fig. S2). In previous studies, the direct conversion of starch to ethanol by a number wild strains of *B. adeninivorans* has been reported (Büttner et al., 1992). More recently, Alok et al. (2016) reported ethanol production from four lignocellulosic waste hydrolysates (bagasse, orange pulp, vegetable waste and wheat straw) by *B. adeninivorans*. These previous studies support the results of our work in that *B. adeninivorans* XE1 has the ability of ethanol fermentation from lignocellulose hydrolysate.

The initial uptake rate of glucose for these two strains was calculated by the change of glucose concentration in the first 6–12 h of the fermentation. IAM 4178 demonstrated a higher initial glucose uptake rate of 0.764 g/L/h (6 h) as compared with 0.0937 g/L/h (6 h) and 0.544 g/L/h (12 h) of XE1. In addition, the ethanol concentration for XE1 was similar to that of IAM 4178, although XE1 degraded both glucose and xylose while IAM 4178 degraded only glucose. Alok et al. (2016) reported that *B. adeninivorans* exhibited lower ethanol yield as compared with *Pichia farinose* and *Stephanoascus ciferrii*. This could explain the lower ethanol fermentation ability of XE1 in the present study.

3.2. Effect of acid hydrolysis conditions on ethanol production

The effect of autoclaving temperature was evaluated by the comparison among Runs 3, 4, and 5 (Fig. 1(c, d, e)). In hydrolysis at 111 °C, the glucose uptake and ethanol production by IAM 4178 were completed within 6 h, and this rate was approximately three times faster than that at 121 °C. Run 5 (XE1) also completely consumed glucose and exhibited a similar ethanol yield as IAM 4178, although the glucose uptake of XE1 was twofold slower than IAM 4178. The reduction of autoclaving temperature from 121 °C to 111 °C remarkably lowered the furfural concentration in the hydrolysate, while the difference in the glucose and xylose concentrations under both conditions was marginal. Schirmer-Michel et al. (2008) also reported that lowering the temperature from 125 °C to 120 °C during acid hydrolysis resulted in significantly lower furfural concentration in soybean hull hydrolysate, with little difference in glucose and xylose concentration. More importantly, it is known that ethanol fermentation resumes once furfural is reduced by yeast (Liu et al., 2004; Taherzadeh et al., 2000). Thus, the faster ethanol fermentation of CS100 hydrolyzed at 111 °C was probably due to the lower initial furfural concentration which resulted in the faster elimination of furfural. From these results, it was inferred that acid hydrolysis at 111 °C was preferable for reducing the production of fermentation inhibitors.

The effect of CS concentration on ethanol fermentation is summarized in Fig. 1(a–g). The hydrolysis efficiency of glucan and xylan, which was calculated by the ratio of glucose or xylose concentration and glucan or xylan content of CS, varied with the dosage of CS and exhibited overall high hydrolysis efficiencies. In low dosage condition (CS20) complete hydrolysis (glucan 96%, xylan 85%) was achieved. In higher CS dosage (CS100 and 200), the hydrolysis of glucan and hemicellulose were slightly less effective at 76–78% and 75–91%, respectively. During ethanol fermentation, with CS100, both yeast strains demonstrated similar ethanol yields. The glucose uptake rate of XE1 was slower than that of IAM 4178, which is a similar trend with CS20. By contrast, CS200 exhibited different ethanol fermentation characteristics for the two yeast strains due to the twofold concentration of not only fermentable sugars, but also furfural (0.784 g/L). For IAM 4178, glucose was completely consumed and yielded 23.6 g/L of ethanol. However, ethanol fermentation by XE1 using CS200 was totally inhibited, showing little uptake of glucose and xylose and the yield of ethanol was only 1.84 g/L. The degradation rate of furfural was much slower than CS100 g/L of XE1 and approximately 0.2 g/L of furfural

still remained at 12 h. These results clearly indicated that ethanol fermentation of XE1 was inhibited by hydrolysis by-products, including furfural. The inhibitory effects of furfural on *S. cerevisiae* have been studied in a number of previous reports (Almeida et al., 2007). These reviewed the inhibitory concentrations and inhibition levels of furfural on *S. cerevisiae*, ranging from 0.8 to 12 g/L and 8–100%, depending on the strains. By contrast, no studies have yet investigated the effect of furfural on *B. adeninivorans* for ethanol fermentation. Alok et al. (2016) reported that the ethanol yield of acid hydrolysate of lignocellulosic waste (bagasse powder, orange peel and pulp, kitchen lignocellulosic waste, and wheat straw waste) fermented by *B. adeninivorans* was lower than that fermented by other yeasts (*Pichia farinose* and *Stephanoascus ciferrii*), despite the fact that the ethanol productivity of *B. adeninivorans* using glucose was similar to that of other yeasts. Accordingly, it could be speculated that *B. adeninivorans* is not so tolerant to furans as compared with other yeasts. From these results and based on its higher tolerance to the inhibitors, IAM 4178 was selected for the following hydrolysate concentration experiment.

3.3. Effect of detoxification on ethanol production

In order to examine the applicability of XE1 for the fermentation of high concentration CS hydrolysate, the effect of detoxification (overliming + activated carbon) on ethanol fermentation by XE1 was evaluated (Fig. 1(h)). The detoxification completely eliminated furfural from the CS hydrolysate, but the glucose concentration also declined from 63.2 g/L to 46.0 g/L. The reduction of glucose could be due to coprecipitation during overliming and/or use of activated carbon. The mechanism of overliming is not yet clarified but a number of papers have reported the reduction of sugar content by overliming alone. Mohagheghi et al. (2006) reported approximately 14% loss of glucose by overliming. Moniruzzaman and Ingram (1998) also confirmed that overliming reduces the glucose and xylose content of rice hull hydrolysate by 70% and 60%, respectively. On the other hand, the effect of activated carbon on lignocellulose hydrolysate has been summarized by Mussatto and Roberto (2004). Silva et al. (1998) dosed activated carbon to sugarcane bagasse hemicellulose hydrolysate at 30% w/w of hydrolysate and reported the sugar reduction by 31.3%. Also, Schirmer-Michel et al. (2008) reported that activated carbon treatment exhibited a loss of glucose (from 1.65 to 1.52 g/L) and xylose (from 30.9 to 28.6 g/L) in soybean hull hydrolysate. In the present study, Run 8 exhibited that the uptake of glucose and xylose and production of ethanol was enhanced but was much slower than those in Runs 5 and 6. Indeed, the complete uptake of glucose took 120 h and xylose was not completely consumed after 192 h in detoxified CS200. More importantly, the produced ethanol concentration of Run 8 was 16.0 g/L, which is a similar value to that of Run 5. From these results, it could be concluded that detoxification is not feasible for the production of high concentration ethanol despite the fact that detoxification alleviates the fermentation inhibition to some extent. As IAM 4178 has a higher tolerance to inhibitors while XE1 cannot produce high concentration ethanol even after application of detoxification procedures, IAM 4178 is suggested to be more feasible for examining the fermentation characteristics of concentrated CS hydrolysate.

3.4. Effect of hydrolysate concentration on ethanol production

The characteristics of ethanol fermentation of concentrated hydrolysate by repeated acid hydrolysis (Run 9) are summarized in Fig. 2. Hydrolysis efficiency of glucan and xylan was 64% and 50% respectively. During ethanol fermentation, a high concentration of furfural (0.943 g/L) was observed in the hydrolysate but this was rapidly degraded and eliminated by 48 h. During this period, glucose uptake and ethanol production were observed. However, the uptake of glucose
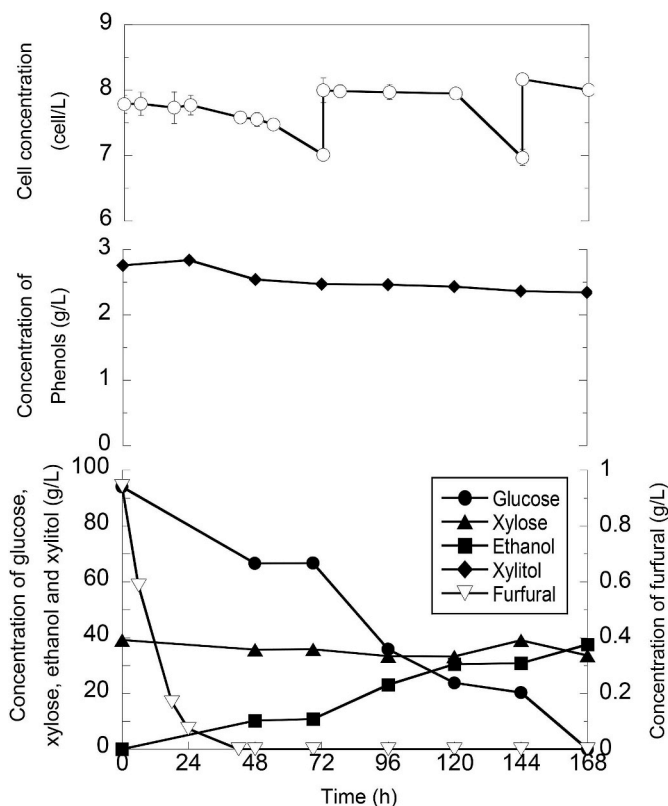
Fig. 2. Time course of IAM 4178 cell density, total phenolics and sugar, furfural and ethanol concentrations during fermentation of concentrated CS hydrolysate by strain IAM 4178. IAM 4178 was intermittently inoculated into the reactor at 0, 72 and 144 h.

ceased by 72 h, and the cell density of IAM 4178 declined to approximately 10% (i.e. 10^7 CFU/mL). Accordingly, IAM 4178 was re-inoculated to maintain the cell density of 10^8 CFU/mL at 72h. After inoculation, the uptake of glucose and the production of ethanol resumed and continued until 120 h. Thereafter, IAM 4178 was re-inoculated again at 144 h, since the production of ethanol ceased and the cell density declined again. As a result, glucose was completely consumed after 168 h, and ethanol concentration eventually reached 37.5 g/L (= 4.8%v/v). The obtained ethanol concentration was 6% lower than the "criterion value" of 40 g/L ethanol. However, glucan content of cassava stem is known to fluctuate between 37.8 and 63.0%-ds (Klinpratoom et al., 2015; Martín et al., 2017; Pooja and Padmaja, 2015), while that of the present study was 39.5%-ds. Accordingly, higher ethanol concentration of 40 g/L or above could be easily obtained depending on the chemical composition of the cassava stem. From these results, it was indicated that intermittent inoculation of IAM 4178 was effective for the fermentation of concentrated acid hydrolysate.

During fermentation, the cell density of IAM 4178 declined by 72 h and by 144 h, although furfural was completely degraded by 48 h. From this result, the existence of other inhibitors together with furfural was suspected. In the present study, the concentration of total phenolics was higher than 2 g/L throughout the fermentation period (Fig. 2). Low molecular weight phenolic compounds, which is often produced by the degradation of phenolic polymer such as lignin or tannin, are known to be toxic to microbial cells, although the mechanism of the inhibiting effect has not been clarified (Palmqvist and Hahn-Hägerdal, 2000b). Delgenes et al. (1996) investigated the effect of phenolic monomers (vanillin, hydroxybenzaldehyde, syringaldehyde) on glucose fermentation of *S. cerevisiae*. They reported that the ethanol yield was 11%, 25%, and 33% of the control (no addition of phenols) in the presence of 2.0 g/L vanillin, 1.5 g/L hydroxybenzaldehyde, and 1.5 g/L

syringaldehyde, respectively. In addition, they observed a significant decline of *S. cerevisiae* cell density (9–19% of the control) by these phenolic monomers. Therefore, it was suggested that the reduction of the cell density of IAM 4178 in Run 9 is probably caused by the presence of phenolic compounds in the hydrolysate. To further improve the ethanol concentration and/or maintain cell density throughout the fermentation period, the phenolic compounds should be removed, such as by applying surfactant (e.g. Tween 20) with high hydrophilic-lipophilic balance (HLB) (Mithra and Padmaja, 2016).

3.5. Ethanol fermentation efficiencies

The ethanol fermentation of all runs was summarized in Table 2, and glucose and furfural concentrations of CS hydrolysate, ethanol concentrations, and glucose-based ethanol fermentation efficiencies (= yield) of CS hydrolysate fermentation using IAM 4178 are summarized in Fig. 3. It was clearly shown that ethanol concentration was proportional to the initial glucose concentration in CS hydrolysates. On the other hand, the ethanol conversion efficiency was similar, with values of 72.4%–82.1%. Since glucose was completely consumed under all conditions, it was suggested that the acid hydrolysis treatment influenced the fermentation time (i.e. glucose uptake rate or ethanol production rate) but did not change the ethanol conversion efficiency. In addition, lowering the autoclaving temperature from 121 °C to 111 °C reduced the production of furfural. These results indicated that the increase of initial sugar concentration under mild acid hydrolysis conditions (i.e. low heating temperature) was preferable for the production of high concentration ethanol from agriculture waste.

Table 2
Summary of ethanol fermentation of cassava stem at different acid hydrolysis condition by strain IAM 4178 and XE1.

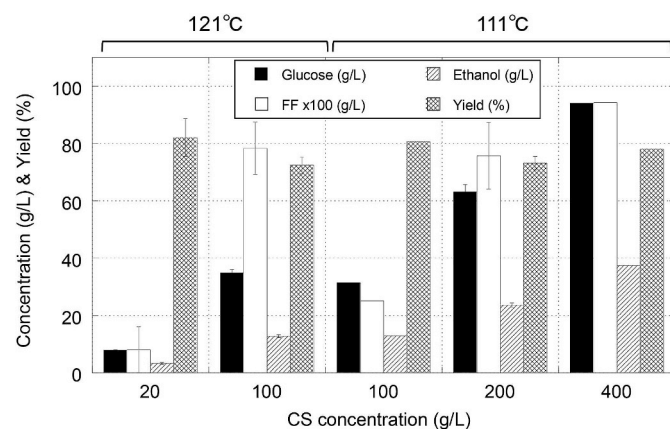| Run	Before fermentation			After fermentation		
	Glucose (g/L)	Xylose (g/L)	Furfural (g/L)	Ethanol (g/L)	Xylitol (g/L)	Furfural (g/L)
1	7.93	2.98	0.08	3.33	0.00	0.00
2	7.93	2.98	0.08	3.06	0.00	0.00
3	34.86	14.61	0.78	12.90	1.15	0.00
4	31.39	16.27	0.25	12.95	1.34	0.00
5	31.39	16.27	0.25	13.55	1.43	0.00
6	63.17	26.26	0.76	23.65	0.65	0.05
7	63.17	26.26	0.76	1.84	0.00	0.01
8	46.06	23.29	0.00	17.42	3.00	0.00
9	94.05	39.12	0.94	37.53	0.00	0.00

Fig. 3. Glucose and furfural concentrations of CS hydrolysate, ethanol concentration, and glucose-based ethanol fermentation efficiencies (= yield) during fermentation of CS hydrolysate by strain IAM 4178.
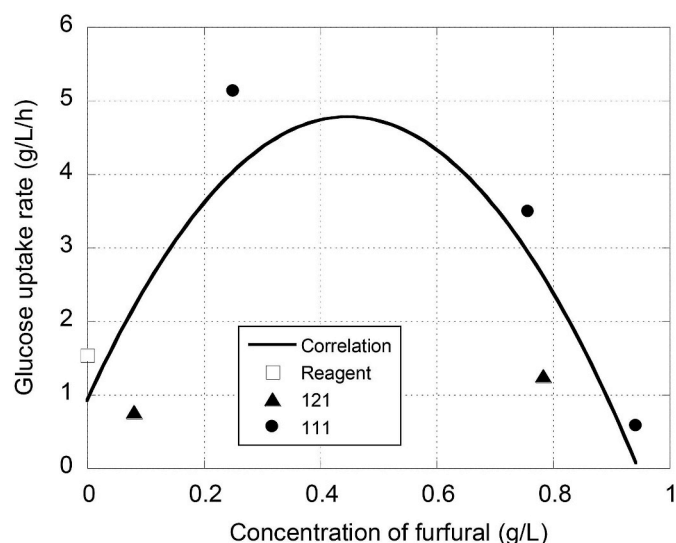
Fig. 4. Relationship between initial glucose uptake rate and furfural concentration during fermentation of CS hydrolysate by strain IAM 4178.

The relationship between initial glucose uptake rate and furfural concentration is summarized in Fig. 4. The results of synthetic medium (glucose + xylose, Fig. S1) is also included for comparing the results of the CS hydrolysate and furfural-free conditions. The glucose uptake rate and furfural concentration exhibited a convex-upward (parabola) relationship. The higher furfural concentration indicates that the acid hydrolysis condition is more severe, which could result in the increase of hydrolysis by-products including furans and phenolic compounds (Palmqvist and Hahn-Hägerdal, 2000a). Severe hydrolytic conditions increase glucose concentration in the hydrolysate such that the initial glucose uptake rate should increase accordingly. However, under high furfural conditions (or severe hydrolytic conditions), the by-products can inhibit ethanol fermentation. Consequently, it was suggested that this convex-upward relationship can be explained by the interactions between enhancement of saccharification and production of inhibitors under severe hydrolytic conditions.

4. Conclusions

The effect of acid hydrolysis on ethanol fermentation of CS was examined to obtain high ethanol production. Two-step acid hydrolysis using concentrated and diluted sulfuric acid at a moderate temperature (111 °C) was preferable for suppressing furfural production. IAM 4178 was more tolerant to the inhibitors as compared with strain XE1. The present study found that the production of high concentration ethanol (37.5 g/L) was achieved by the fermentation of concentrated CS hydrolysate by two-times hydrolysis and the intermittent inoculation of IAM 4178.

Conflicts of interest

The authors declare no conflicts of interest associated with this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2018.12.007.

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