Simultaneous co-saccharification and fermentation of sago hampas for bioethanol production

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Abstract: Abundance of lignocellulosic biomass provides a good solution to the demands of energy crops in producing biofuel like biodiesel and bioethanol. In this study, bioethanol was produced from sago hampas via the Simultaneous co-Saccharification and Fermentation (Sc-SF) process, at 2.5% and 5.0% (w/v) solid loadings. The processing step in Sc-SF is virtually similar to that of Simultaneous Saccharification and Fermentation (SSF). However, during Sc-SF, two enzymes, amylase and cellulose, were added for the co-saccharification of sago starch and fiber. In addition, Saccharomyces cerevisiae was used to ferment the sugars in the hydrolysates. The Sc-SF samples were analyzed for carbohydrate residues, ethanol and acetic acid using the dinitrosalicylic (DNS) acid assay and High Performance Liquid Chromatography (HPLC).Results showed that the Sc-SF of the sago hampas showed high efficiencies of hydrolysis and ethanol production within the first six hours of fermentation. Highest glucose production was at 37.86 g/l for the 5.0% sago hampas load and 17.47 g/l for 2.5% sago hampas load. The highest ethanol production was observed in the broth with 5.0% sago hampas, with a theoretical yield of 80.50%. Meanwhile, the highest bioethanol yield in the sample with 2.5% sago hampas was 73.19%. This study indicated that bioethanol production via Sc-SF from starch rich agricultural residues such as sago hampas is feasible.

Keywords:bioethanol, sago hampas, simultaneous co-Saccharification and Fermentation (Sc-SF), amylase, cellulase, Saccharomyces cerevisiae, HPLC

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1 Introduction

The demand on fossil fuels for energy has increased exponentially since the explosion of industries in the first world and developing countries and the increase is predicted to continue (Sun and Cheng, 2002; Karki et al., 2012; Vincent et al., 2014). On the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050 (Campbell and Laherree, 1998). Together with the continual fluctuation in oil prices, this phenomenon has sparked a renewed interest in the potential use of renewable sources such as lignocellulose to produce a variety of liquid biofuels such as biodiesel and bioethanol (Vincent et al., 2014). The current leading nations in bioethanol production are USA and Brazil whereas Asian countries altogether account for about 14% of world's bioethanol production (Carere et al., 2008; Vincent, 2010).

Historically, biofuel productions are basically categorized into two phases, first and second generation. First generation biofuels are produced primarily from food crops. In Brazil, about 70% of ethanol is produced from fresh sugarcane and the remaining percentage is from cane molasses (Wilkie et al., 2000). Meanwhile, bioethanol in USA is produced almost exclusively from corn (Vincent et al., 2011b). The main concern regarding first generation biofuels is the impact biofuel production may have on land biodiversity and the competition with food crops (Pimentel and Patzek, 2005; Mitchell, 2008).

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Thus, undefined long term viability of bioethanol from first generation bioprocesses has led to researchers focusing second generation processes (Vincent, 2010).

Second generation biofuels are produced primarily from lignocellulosic biomass (Vincent et al., 2011a). These resources are widely abundant, comprising approximately 50% of the world annual biomass production, estimated at 10-50 billion tons (Claassen et al., 1999). Second generation biofuels are expected to provide both the short term benefits of first generation biofuels, as well as many other long term benefits (Vincent et al., 2011b). Therefore, the production of bioethanol from lignocellulosic biomass, especially agricultural and industrial residues, is clearly one of the best energy alternatives.

In Sarawak, Malaysia, agro-residues from sago starch processing industries are abundant, readily available and cheap. It is estimated that approximately seven tons of sago pith wastes are produced daily from a single sago starch processing mill (Awg-Adeni et al., 2010). These residues are deposited in the factory compound, and are often washed out into nearby streams together with the factory wastewater. Sago wastewater consists of high organic loads, both solid and liquid. The solid portion of the waste is known as sago hampas. According to Linggang et al. (2012), sago hampas contains approximately 58.0% starch, 23.0% cellulose, 9.2% hemicellulose, and 4.0% lignin on a dry weight basis, making it a potential feedstock for bioethanol production via second generation processes. Therefore the present study was done to produce bioethanol from ground sago hampas via the Simultaneous co-Saccharification and Fermentation (Sc-SF) process using amylase, cellulose and the yeast, *Saccharomyces cerevisiae*.

2 Materials and methods

2.1 Experimental setup

Figure 1 shows the flowchart of the experimental setup. Experiments were performed in duplicate (n=2) and analyses were conducted in triplicates (n=3).

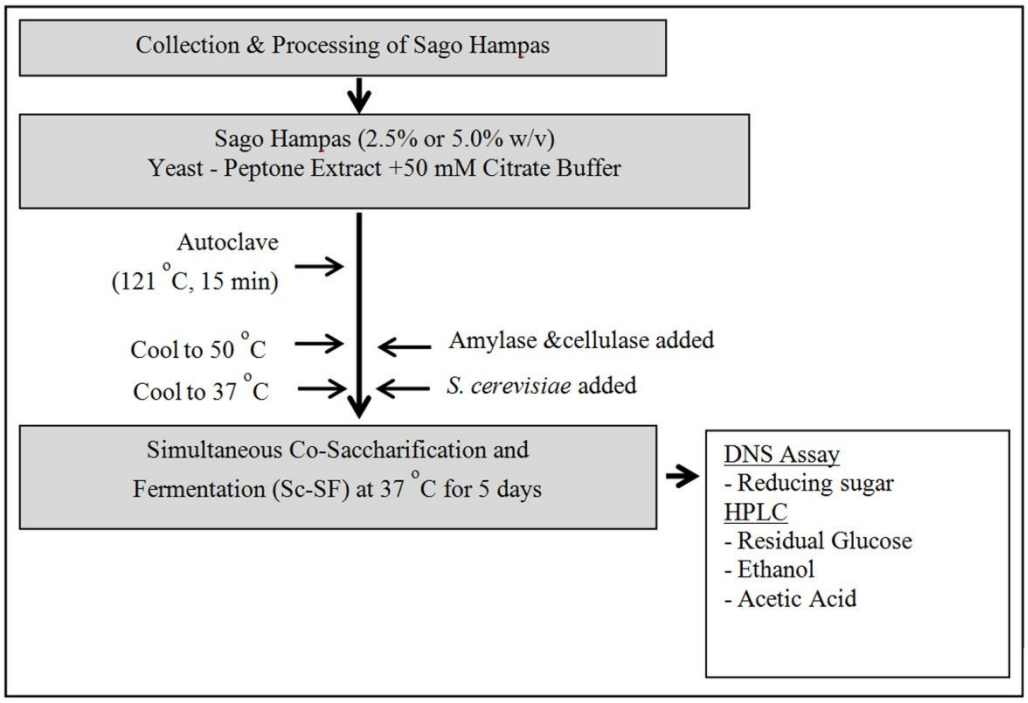

Figure 1 Flowchart of process outlining the steps for Simultaneous co-Saccharification and Fermentation (Sc-SF) of sago hampas (2.5% and 5.0%) using amylase, cellulase and *S.cerevisiae*.

2.2 Sample collection and preparation of fermenting microorganisms

Sago hampas was obtained from Pusa, Sarawak. The hampas was dried and ground to pass a 1 mm screen.

Saccharomyces cerevisiae (ATCC 24859) used in this study was obtained from the American Type Culture Collection (Rockville, MD). The *S. cerevisiae* inoculum was prepared by growing the culture overnight in 100 ml of sterile LB broth at 32 ℃ with constant agitation at 120 r/min. The *S. cerevisiae* cells were then harvested via centrifuge in two 50 ml conical centrifuge tubes for five minutes at 6000 r/min (Vincent et al., 2011a).

2.3 Simultaneous co-Saccharification and Fermentation (Sc-SF)

Simultaneous co-Saccharification and Fermentation (Sc-SF) was carried out in 250 ml bottles with final working volumes of 150 ml. The sago hampas for Sc-SF was mixed with 50 mM Citrate Buffer –Yeast Peptone (CB-YP) solution and was sterilized at 121 ℃ for 15 min and kept in a 50 ℃ oven prior to the addition of amylase (5.56 U/ml) (Dextrozyme® GA, Novozymes A/S, Bagsvaerd, Denmark) and cellulase (50 FPU/g cellulose) (Accellerase® 1000, Genencor, CA, USA). Each batch of fermentation was prepared in duplicates (n=2). After the addition of the enzymes, the fermentation broth was kept in a 37 ℃ incubator with agitation at 150 r/min for about one to two hours. Once the temperature of the fermentation broth has decreased to 32 ℃-37 ℃, harvested 24 hours old *S. cerevisiae* culture (10^8 CFU/ml) was inoculated aseptically. The fermentation was carried out under anaerobic condition for five days at 37 ℃ with a constant agitation of 150 r/min. Samples were collected at 0, 6, 12, 24, 48, 72, 96 and 120 h respectively. All samples were filtered through a 0.2 μm nylon syringe filter (Whatman, NJ, USA) prior to DNS dan HPLC analyses.

2.4 Dinitrosalicylic (DNS) acid reducing sugar assay

The filtered supernatant from the fermentation broth was tested for free reducing sugar via the dinitrosalicylic (DNS) acid assay (Miller, 1959). Pre-dilution was done to obtain absorbance values within acceptable range. Filtered supernatant from 2.5% sago hampas was diluted with ddH_2O in the ratio of 1:2, while filtered supernatant from 5.0% sago hampas was diluted in the ratio of 1:4. Color formation in the DNS assay was determined by measuring absorbance against the reagent blank at 540 nm and the absorbance value was converted into equivalent sugar concentration based on a standard glucose curve constructed previously (Vincent, 2010). All the analyses were performed in triplicates (n=3).

2.5 High performance liquid chromatography (HPLC)

The soluble compounds were analyzed using High Performance Liquid Chromatography (HPLC; Shimadzu, Kyoto, Japan) using a refractive index detection system. Filtered samples from the Sc-SF were analyzed for the presence of glucose, ethanol and acetic acid on Aminex HPX- 87H column (Bio-Rad, Chemical Division, Richmond, CA).The separation and analysis of fermentation products constituents was done on a Bio-Rad Aminex HPX-871 column (150 × 7.8 mm; Bio-Rad Chemical Division, CA, USA) using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.8 ml/min and a 20 μl injection volume (Vincent et al., 2011b; Vincent et al., 2014).The column temperature was maintained at 65^{o}C (Vincent et al., 2011a).

3 Results and discussion

This study was done to determine the feasibility of ethanol production from sago starch residue and sago fiber via Simultaneous co-Saccharification and Fermentation. Ethanol was produced immediately by the yeast *Saccharomyces cerevisiae* following the saccharification of the starch and cellulose. From Figure 2, ethanol production increased rapidly between 6 to 12 h during Sc-SF in both concentrations of sago hampas. The highest theoretical yield of bioethanol recorded at 24 h in the fermentation broth with 5.0% sago hampas was 80.5%, while the highest theoretical yield of bioethanol for fermentation using 2.5% sago hampas was 73.2%. At 0 h, the glucose readings were high at 14.76 g/l and 31.67 g/l in the broth containing 2.5% and 5.0% sago hampas, respectively. These glucose concentrations were the result of the initial saccharification of the starch by the amylase added when temperature of the broths were at 50^{o}C. At 6

h, glucose concentration increased slightly for all samples to 17.47 g/l (2.5% sago hampas) and 37.86 g/l (5.0% sago hampas). After 6 h, the results showed rapid drop in glucose concentration, followed by rapid increase of bioethanol production at 12 h. Generally, glucose and ethanol production would increase during Sc-SF as the substrate load was higher, however, this is limited to fermentation with 5.0% to 9.0% (w/v) substrate load (Awg-Adeni et al., 2012). Substrate loading beyond 9.0% (w/v) is reported to significantly reduce conversion yields as the increasing viscosity of the fermentation broth causes inefficient enzymes to substrate contacts, as well as, poor heat transfer (Gupta et al., 2012).

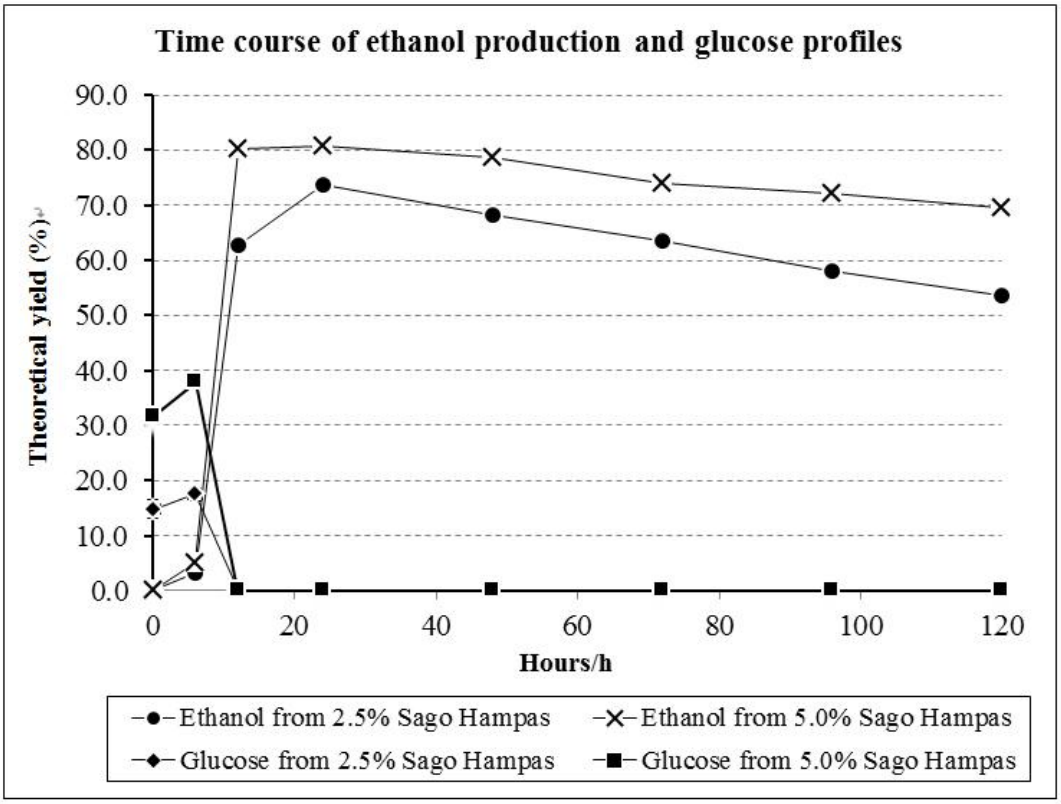

Figure 2 Time course of ethanol production and glucose profiles during Sc-SF, as determined via HPLC. The data points represent the averages of two independent experiments (n=2)

High ethanol yield in our study was made possible with the Sc-SF process. Sc-SF is a modified version of Simultaneous Saccharification and Fermentation (SSF). The sequence of steps in Sc-SF is the same as the SSF, except for the co-saccharification of sago starch residue and sago hampas prior to the fermentation process by *S. cerevisiae*(Vásquez et al., 2007). While there is a reported attempt to produce bioethanol from sago starch (Awg-Adeni et al., 2013), none has been made on the simultaneous co-saccharification of the starch and fiber in sago hampas. In this study, co-saccharification was achieved using commercial amylase and cellulase.

Efficiencies of the co-saccharification step are crucial in the production of bioethanol via Sc-SF as more fermentable sugars (i.e. glucose, xylose, arabinose and galactose) released would be converted into more ethanol. However, due to the limitation of the organic acid column used in our HPLC analyses to detect and differentiate certain mono-, di- and oligosaccharides, DNS reducing sugar assay was performed to complement the carbohydrate reading. Figure 3 shows the concentration of reducing sugar throughout the Sc-SF period. The highest concentration of reducing sugar was detected at 6 h, with the concentration of 25.37 g/l and 51.33 g/l for 2.5% sago hampas and 5.0% sago hampas, respectively. This was followed by a sharp decrease at 12 h of Sc-SF, from 51.33 g/l to 4.10 g/l for 5.0% sago hampas. Similarly, reducing sugar concentration dropped from 25.37 g/l to

4.20 g/l for 2.5% sago hampas. At 12 to 120 h, the concentration of reducing sugar in both fermentations was almost constant, between 4.2 g/l to 1.31 g/l for 2.5% sago hampas and 4.10 g/l to 3.52 g/l for 5.0% sago hampas. This drastic decrease, followed by near absence of reducing sugar during fermentation is a common observation when working with starch biomass such as sago hampas to produce bioethanol, as this simply indicates that conversion of glucose, or other reducing sugar, are at optimum level(Awg-Adeni et al., 2013).

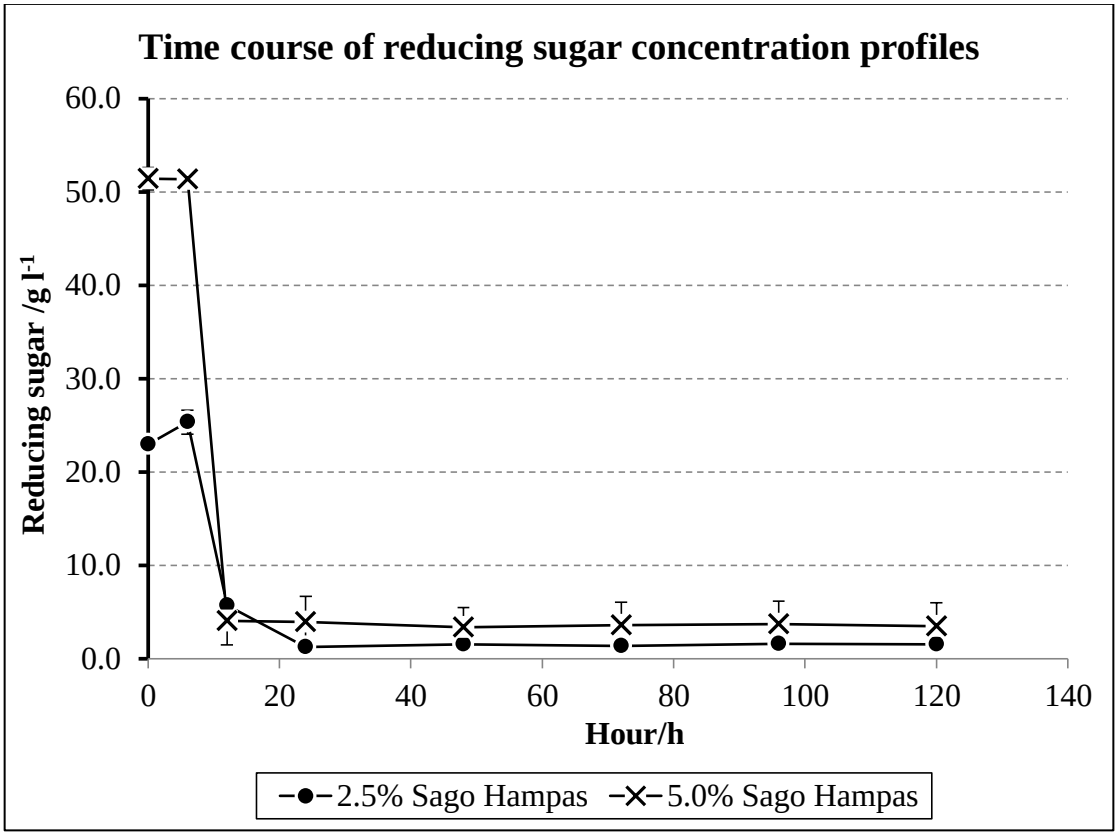

Figure 3 Time course of reducing sugar concentration profiles during Sc-SF, as determined via the DNS assay. The data points represent the averages of two independent experiments (n=2).

According to Chew and Shim (1993), a large number of starch granules are trapped within the lignocellulosic matrix of sago hampas. Therefore, prior to the saccharification process, the sago hampas underwent gelatinization at 121℃ for 15 min. Gelatinization is the swelling of the starch granule in the presence of heat and water. This condition increases the efficiency of enzymatic hydrolysis on the polysaccharides as more surface areas are exposed (Awg-Adeni et al., 2010; Awg-Adeni et al., 2013). Upon gelatinization, pullulanase from *Trichodermareesei* and glucoamylase from *Aspergillus niger* were introduced to hydrolyze residual starch in the sago hampas to fermentable glucose monomers to be initially fermented into ethanol. Pullulanase is an endo-amylase which acts as debranching enzymes to hydrolyze α-1, 6 links in the chains, forming dextrins, while glucoamylase is an exo-amylase that hydrolyzes both maltose and dextrin from the non-reducing end of the molecule to form glucose (Montalbo-Lomboy et al., 2008).

Enzymatic hydrolysis of lignocelluloses constituent was carried out by cellulase enzymes. Usually, the lignocellulosic materials have to be pretreated before enzymatic hydrolysis. According to Lynd (1996), the yield of cellulose hydrolysis often exceeds 90% of the theoretical value when pretreatment was carried out. Pretreatment can be either via chemical (acids, alkaline solutions) or physical (heat, size reduction) means

(Vincent, 2010). Therefore, in this experiment sago hampas was ground and sieved through 1 mm screen. The commercial cellulase utilized in this study was produced from genetically modified *T. reesei*, consisting of ß-1-4-endoglucanase, ß-1-4-exoglucanase, and ß-glucosidase. ß-1-4-endoglucanases attacks regions of low crystallinity in the cellulose fiber, creating free chain ends. Then, ß-1-4-exoglucanase degrades the molecules further by removing cellobiose units from the free chain ends. Finally, ß-glucosidase hydrolyzes cellobiose to produce glucose(Vincent, 2010).

Another indication of efficient biomass hydrolysis is the increase in acetic acid concentration in the fermentation broth. The monitoring of acetic acid is crucial as this organic acid has the ability to direct several glycolytic intermediates to the corresponding metabolic pathways, ultimately, decreasing ethanol yield (Wyman et al., 2005). Acetic acid is usually derived from the hydrolysis of the acetyl groups bound to the hemicellulosic monomers(Aguilar et al., 2005; Wyman et al., 2005). As more hemicellulose is degraded, increasing amount of acetic acid is detected, as supported by previous studies (Aguilar et al., 2005; Vincent et al., 2011b).This is also seen in our study as is shown in Figure 4. Acetic acid was produced rapidly during Sc-SF with 2.5% sago hampas once *S. cerevisiae* was inoculated. Within 12 h of fermentation, the concentration increases from 0 g/l to 0.12 g/l. Rapid production of acetic acid was also recorded at day 1 where the concentration increases from 0.12 g/l to 0.40 g/l. Sc-SF with 5% sago hampas also produced detectable amount acetic acid. However, contrary to 2.5% sago hampas, the production increases slowly, from 0 g/l to 0.02 g/l in the fermentation broth with 5.0% sago hampas. For all samples, the concentration increased steadily until the final day of fermentation.

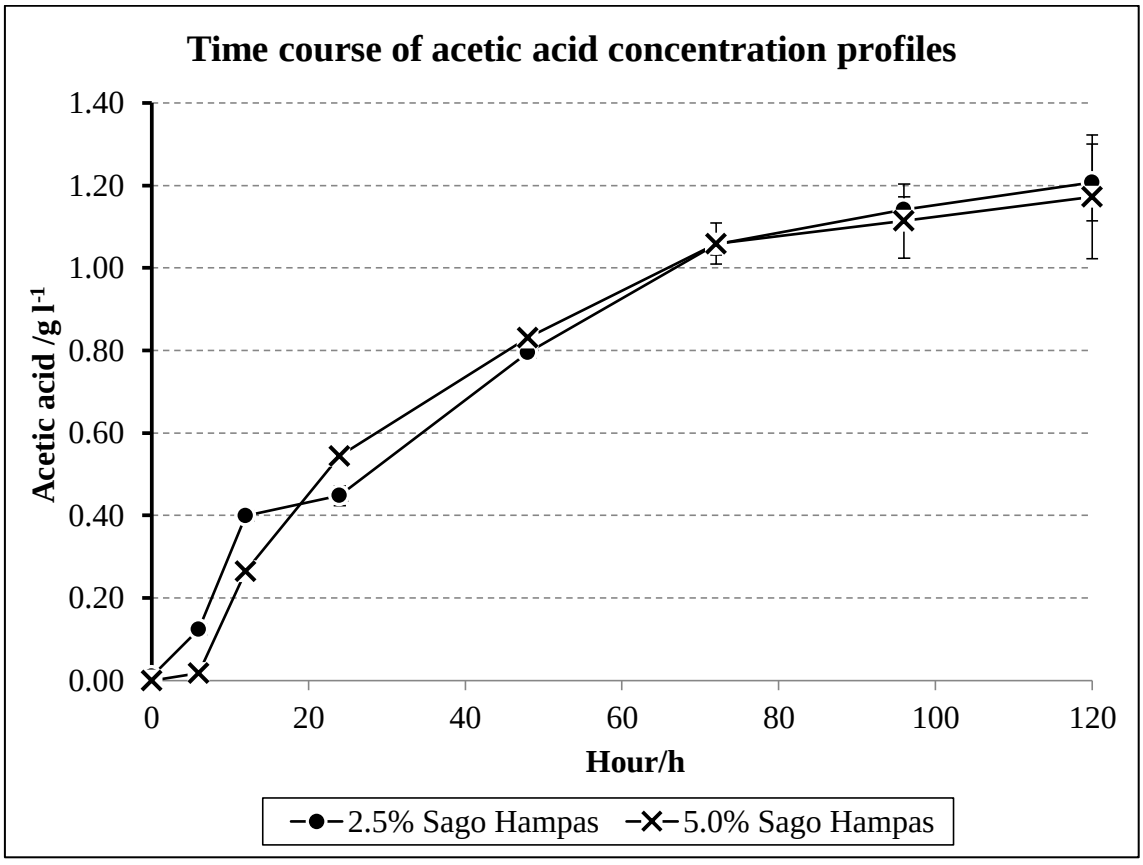

Figure 4 Time course of acetic acid concentration profiles during Sc-SF, as determined via HPLC. The data points represent the averages of two independent experiments (n=2)

4 Conclusions

In conclusion, sago hampas can be utilized in the production of bioethanol via Simultaneous co-Saccharification and Fermentation (Sc-SF). For Sc-SF using 5.0% sago hampas feedstock, the highest concentration of total reducing sugar produced from enzymatic hydrolysis using amylase and cellulase was 51.33 g/l, while the highest glucose concentration was 37.86 g/l. Both concentrations were highest at 6 h. Meanwhile, the highest concentration of total reducing sugar produced in Sc-SF using 2.5% sago hampas was 25.37 g/l and the highest glucose concentration was 17.47 g/l. Similarly, both concentrations were highest at 6 h. Hydrolysates produced through co-saccharification were fermented by *S. cerevisiae* and the highest theoretical yield of bioethanol in 5.0% sago hampas was 80.50%at 12 h. Fermentation by *S. cerevisiae* in fermentation broth with 2.5% sago hampas produced bioethanol with the highest theoretical yield of 73.19%, also at 12 h.

Acknowledgements

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