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Production of bioethanol from sago hampas via Simultaneous Saccharification and Fermentation (SSF)

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Abstract. *Huang CH, Adeni DSA, Johnny Q, Vincent M. 2018. Production of bioethanol from sago hampas via Simultaneous Saccharification and Fermentation (SSF). Nusantara Bioscience 10: 240-245.* Sago hampas is an inexpensive, renewable and abundant agro-industrial residue that can be exploited to produce bioethanol. In this study, ethanol production was performed via simultaneous saccharification and fermentation (SSF) on fresh sago hampas at 2.5%, 5.0% and 7.5% (w/v) feedstock loadings with the aid of amylolytic enzymes, cellulolytic enzymes and *Saccharomyces cerevisiae*, under anaerobic condition for five days with a constant agitation of 150 rpm and ambient temperature. Results obtained indicated that SSF with 5.0% (w/v) sago hampas loading produced the highest ethanol yield at 17.79 g/L (79.65% Theoretical Ethanol Yield, TEY), while SSF using 2.5% and 7.5% (w/v) sago hampas produced ethanol at only 8.38 g/L (75.00% TEY) and 23.28 g/L (69.48% TEY), respectively. Total biomass reduction was recorded between 66.3% to 71.3% by the end of the SSF period. This study demonstrated that fresh sago hampas is a promising feedstock for bioethanol production as yields are generally high for all the substrate loadings tested. Moreover, bioethanol production using fresh sago hampas may assist in reducing pollution caused by sago waste accumulation.

Keywords: Bioethanol, cellulose, enzymatic hydrolysis, sago hampas, simultaneous saccharification and fermentation, SSF

INTRODUCTION

Fuel demands from the transportation sector, which is the biggest user of diesel and gasoline, has increased tremendously in a worrying trend (Aditiya et al. 2016). These fossil-based fuels are not only non-renewable, but they are also not environmentally, ecologically or economically sustainable in a long term (Vincent et al. 2015a; Adekunle et al. 2016; Zabed et al. 2014). Therefore, alternative liquid biofuel, such as bioethanol, is currently being mass produced to substitute and supplement petroleum-based fuel due to its sustainability and carbon dioxide neutrality (Vincent et al. 2014). However, the current practices of using food and feed based feedstock, such as corn and sugar cane, are undesirable and controversial due to the concerns on food security (Daylan and Ciliz 2016). Therefore, the utilization of non-edible lignocellulosic materials for second-generation bioethanol production is capturing the attention of many researchers due to the restrictions in the use of food crops for first-generation bioethanol production as well as the high costs involved (Aditiya et al. 2016; Daylan et al. 2016).

Non-edible lignocellulosic materials are composed of cellulose, hemicellulose and lignin. These materials include waste streams from various sources such as food processing industries, forestry, agriculture, domestic and municipality origins. Additionally, starch is also found in several starchy-lignocellulosic biomasses such as cassava pulp, food waste and sago hampas (Behera et al. 2014; Thangavelu et al. 2016; Wan et al. 2016). These resources do not entirely compete with agricultural crops, and, are excellent candidates to be used as raw materials for bioethanol production (Tye et al. 2016).

Sarawak is the largest sago-growing region in Malaysia and has been recognized as the biggest sago starch exporter in the world. Sarawak exports approximately 44,000 tonnes of starch annually, mainly to Peninsular Malaysia, Japan and Singapore (Awg-Adeni et al. 2013). However, the production of this precious commodity also generates wastes, in the forms of organic rich effluent and solid residues called sago hampas, that are discarded into nearby streams, as well as deposited in the factory compound (Awg-Adeni et al. 2010). These practices have led to several environmental issues, such as water pollution and wastage of valuable resources (Wan et al. 2016). Sago hampas is made of 58% starch, making it relatively easier and less expensive to process compared to another lignocellulosic biomass (Vincent et al. 2015b). It can be found in abundance in a typical sago starch processing plant where an estimated 7 tonnes of sago pith waste are generated daily (Awg-Adeni et al. 2010).

Therefore, in order to find a possible use for this misplaced solid residue, this study was performed to investigate bioethanol production from sago hampas at several loadings via simultaneous saccharification and fermentation (SSF) with the aid of commercial amylolytic and cellulolytic enzymes, in the presence of *Saccharomyces cerevisiae*. According to Vincent et al. (2015a), it is a preferred method for the production of second-generation bioethanol from lignocellulosic biomaterials. During SSF, resultant sugars liberated by the hydrolyzing enzymes are simultaneously metabolized by fermenting microorganisms

to produce ethanol, thereby alleviating problems caused by product inhibition (Awg-Adeni et al. 2013; Kannan et al. 2013; Vincent et al. 2015b). Other advantage of using SSF include lower enzyme dosages, higher ethanol yields, lower requirement for sterile conditions, shorter processing time and cost reduction by eliminating expensive reaction and separation equipment (Jessen et al. 2012; Kang et al. 2014; Triwahyuni et al. 2015).

MATERIALS AND METHODS

Characterization of sago hampas

Sago hampas was collected from a local sago starch processing plant in Pusa, Sarawak (Malaysia). The sample moisture content was determined by weight loss from initial weight after oven drying at 105°C for 3 days. Prior to compositional analysis, sago hampas was destarched and dried for 3 days before it was ground to pass through a 1 mm (20 mesh) screen sieve. The samples were subjected to starch content and fibre analysis using phenol sulphuric acid assay, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF), Klason Lignin Determination according to Goering and van Soest (1970). Ash content was determined by ashing samples at 500°C for 24 h. All analyses were performed in triplicates (n=3).

Saccharomyces cerevisiae culture preparation

Saccharomyces cerevisiae ATCC 24859 culture inoculum for fermentation was prepared by growing the culture overnight in sterile Yeast Malt (YM) broth, in 250 mL conical flask with constant agitation at 120 rpm. The cell pellets were harvested through centrifugation in 50 mL falcon tube for 6 min at 4,500 rpm. After discarding the supernatants, the cells were added into the fermentation broth during the initial stages of the simultaneous saccharification and fermentation (SSF).

Simultaneous Saccharification and Fermentation (SSF)

SSF were performed in 500 mL Schott bottles with batch cultures of 200 mL, consisting of 50 mM Yeast Peptone Citrate Buffer (YP-CB) and sago hampas (2.5%, 5.0% and 7.5% w/v). The fermentation broth was autoclaved to provide sterility and to gelatinise the starch. After that, broths were kept in a 50 °C oven prior to the addition of amylases (12.5 U/mL of α-amylase; 25.0 U/mL of glucoamylase) and cellulase (50 FPU/g cellulose). Once the temperature of the fermentation broth had cooled down to ambient temperature, the harvested *S. cerevisiae* cells were inoculated aseptically. SSF was performed under anaerobic condition for five days with a constant agitation of 150 rpm at ambient temperature. Sample aliquots of 1.5 mL samples were pipetted aseptically from each bottle at predetermined time intervals and centrifuged at 13,500 rpm for 5 min. The supernatant was then filtered through 0.45 µm nylon syringe filter prior to analysis. At the end of fermentation period, the remaining biomass was centrifuged and dried for determination of biomass reduction.

Phenol-sulphuric total carbohydrate assay

The filtered supernatant was subjected to identify the total carbohydrate via phenol-sulphuric acid (PSA) assay (Crawfod and Pometto 1988). Colour formation in the PSA assay was determined by measuring absorbance against the reagent blank at 490 nm and the absorbance value was converted into equivalent values based on a standard curve constructed. All the analyses were performed in triplicate (n=3).

High Performance Liquid Chromatography (HPLC) analysis

Filtered sample aliquots were also analysed using high performance liquid chromatography (HPLC) for residual glucose, maltose/cellobiose and xylose as well as fermentation products such as ethanol and acetic acid. The HPLC system (LC-20A, Shimadzu, Japan) used in this study was equipped with a column oven (CTO-20A, Shimadzu, Japan), refractive index detector (RID-10A, Shimadzu, Japan), isocratic pump (LC-20AT, Shimadzu, Japan) and computer controller. The separation and analysis of ethanol and other fermentation constituents were done on a Bio-Rad Aminex HPX-8711 column (150 × 7.8 mm; Bio-Rad, CA, USA). Filtered aliquots of 20 µl were injected into HPLC using 5 mM H_2SO_4 as the mobile phase, operating at a rate flow of 0.8 mL/min and the column was heated to 65 °C (Vincent et al. 2015b).

RESULTS AND DISCUSSION

Characterization of sago hampas

This study was conducted to utilize waste sago hampas to produce bioethanol via simultaneous saccharification and fermentation (SSF) at 2.5%, 5.0% and 7.5% (w/v) solid loadings. Prior to the SSF process, compositional analyses were done on the sago hampas fibre. The results, as presented in Table 1, show that the main components in dried sago hampas are starch (55.40±0.02%), cellulose (23.64±0.77%), hemicellulose (9.07±1.18%), lignin (4.01±0.51%) and ash (2.23±0.01%). The high percentages of starch and cellulosic components are comparable to the previous studies by Awg-Adeni et al. (2013), Thangavelu et al. (2014) and Vincent et al. (2015b), which were almost similar. These observations suggest that sago hampas is a choice material for amylo-cellulolytic hydrolyses to be further used downstream for ethanolic fermentation by fermenting microorganism such as S. cerevisiae (Arapoglou et al. 2010).

Table 1. Compositional analysis of sago hampas (as percentages based on dry weight; n=3)

Chemical composition	% w/w
Starch	55.4 ± 0.02
Cellulose	23.6 ± 0.77
Hemicellulose	9.1 ± 1.18
Lignin	4.0 ± 0.51
Ash	2.2 ± 0.01
Others	5.7 ± 0.09

Simultaneous saccharification and fermentation (SSF)

Enzymatic saccharification is an important step prior to fermentation in yielding as much fermentation sugars as possible for the microbial conversion into ethanol. In this process, amylolytic enzymes, α-amylase and glucoamylase were used as both α-1,4 and α-1,6- debranching hydrolases efficiently break down gelatinized starch into maltose and glucose, for posterior saccharification and fermentation processes (Aydemir et al. 2014). These amylolytic enzymes act synergistically during the successive enzymatic saccharification of starch in accordance to several previously reported studies (Kannan et al. 2013; Diong et al. 2016). Later, cellulolytic enzymes, consisting mainly of β-1,4-endoglucanse, β-1,4-exoglucanse and β-glucosidase, were added to disrupt the solid fibre structure at the solid-liquid interaction, making the individual fibres available for hydrolysis (Lakhundi et al. 2015).

Phenol-sulphuric total carbohydrate assay

Total carbohydrate concentration in this study was determined via phenol-sulphuric acid assay and the results are depicted in Figure 1. The initial total carbohydrates for 2.5%, 5.0% and 7.5% (w/v) sago hampas loadings were 18.26, 39.55 and 56.53 g/L, respectively. Throughout the fermentation period, the total carbohydrates in the batch system of 2.5% (w/v) feedstock loading decreased steeply in the first 6 h and then remained virtually constant. Meanwhile, the other two feedstock loadings decreased sharply in the first 12 h. Based on the results obtained, experiments with 7.5% (w/v) substrate showed the highest carbohydrates consumption of 87.0% (49.16 g/L) compared to the other substrate concentrations. However, residual carbohydrates were still detected in all fermentation media on the final day of SSF, indicating that not all resultant sugars were converted into ethanol.

High performance liquid chromatography (HPLC) analysis

To supplement the carbohydrate assay, the efficiency of enzymes in hydrolysing the sago hampas were gauged and validated through HPLC analysis for the presence of the main monomeric carbohydrate end products from starch and cellulose (Vincent et al. 2014). Figure 2 shows that the glucose concentration profiles were similar for the all feedstock loadings. The initial glucose production in the broth with 5.0% (w/v) sago hampas loading showed a two-fold higher reading than 2.5% (w/v) sago hampas loading, which was 34.72 g/L compared to 18.29 g/L. However, at 0 h, the glucose concentration in the broth with 7.5% (w/v) sago hampas loading was valued at 42.83 g/L only, due to the inefficient enzymatic saccharification of starch (Gupta et al. 2012; Vincent et al. 2015b). By 18 h, no glucose was detected in all broth samples; a good indication that efficient conversion of glucose into ethanol was achieved during fermentation.

Maltose/cellobiose are other saccharification products from starchy-cellulosic materials (Su et al. 2010; Bhaumik and Dhepe 2015). The initial maltose/cellobiose concentrations (Figure 3) were between 1.03 to 1.54 g/L for all feedstock loadings. By 6 h, early depletion of maltose/cellobiose was detected in the fermentation with 2.5% (w/v) sago hampas loading. In contrast, maltose/cellobiose concentrations peaked at 2.22 and 2.74 g/L for experiments with 5.0% (w/v) and 7.5% (w/v) sago hampas loads, respectively. The levels then decreased sharply until no maltose/cellobiose was further detected by 18 h, demonstrating the effectiveness the enzymatic conversion of maltose/cellobiose to glucose and promptly into ethanol by yeast (Ferreira et al. 2010).

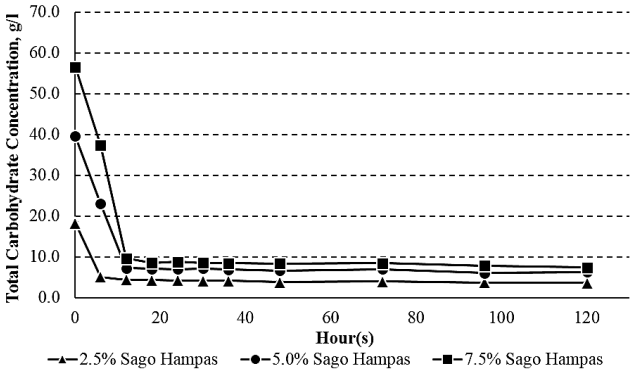

Figure. 1. Time course of total carbohydrate concentrations, as determined by the phenol-sulphuric acid assay (n=3)

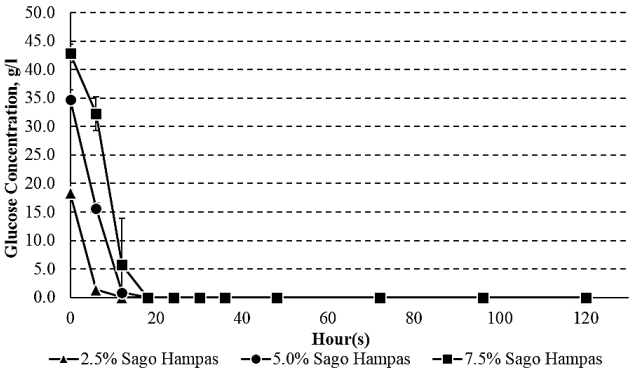

Figure 2. Time course of glucose concentrations, as determined via HPLC

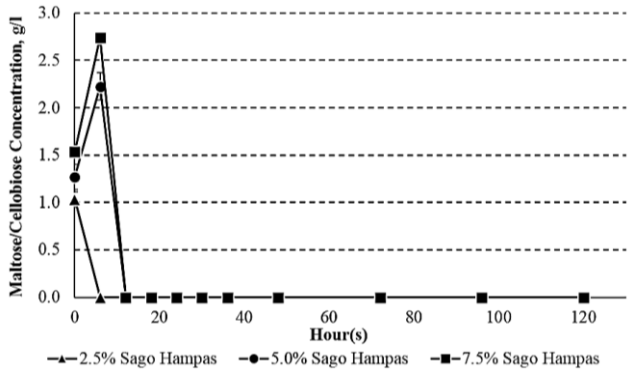

Figure 3. Time course of maltose/cellobiose profile during SSF, as determined via HPLC

Effective enzymatic hydrolysis of a typical lignocellulosic material, such as sago hampas, can be gauged not only by the glucose and maltose/cellobiose profiles, but also by the amount of xylose released into the fermentation broth (Im et al. 2016). This is anticipated as xylanases are part of the enzyme consortia used. The presence of xylanases is also vital as xylanases have synergetic effect on biomass hydrolyses by degrading heterogenous xylan polymers that surround cellulose microfibrils, resulting in enhanced hydrolyses of the overall lignocellulosic biomass (Wi et al. 2013). Figure 4 shows the xylose production profiles. In general, xylose production increased throughout the SSF period. These observations could be explained by the inability of *S. cerevisiae* to efficiently utilise xylose as a carbon source and its conversion into ethanol (Cassa-Barbosa et al. 2015; Srivastava et al. 2015).

The highest theoretical ethanol yield (TEY) was recorded in broth with 5.0% (w/v) sago hampas loading at 79.65% or 17.79 g/L at 48 h (Figure 5). This is significantly higher than TEY in the samples with 2.5% and 7.5% (w/v) sago hampas loadings, which were 75.00% TEY (8.38 g/L) and 69.48% TEY (23.38 g/L), respectively. For the experiment with 2.5% (w/v) sago hampas loading, ethanol concentration increased sharply to 75.00% TEY from 6 to 12 h, decreasing marginally thereafter until 120 h to 62.38% TEY. At 7.5% (w/v) sago hampas loading, theoretical yield of bioethanol production increased sharply to 61.77% TEY from 0 to 12 h, increasing slightly until reached its maximum ethanol production peaked at 48 h.

Based on the TEY profile in Figure 5, one notable observation was that the differences in peak ethanol productions in all fermentation broths occurred inversely to the fermentable sugars concentrations present in the fermentation media, as reported elsewhere (Vincent et al. 2015a). The concentration of glucose (Figure 2) was higher at 7.5% (w/v) sago hampas and longer duration was required for the *S. cerevisiae* to fully convert glucose to ethanol, while, the broth with 2.5% (w/v) sago hampas had lower fermentable sugars concentrations, requiring a shorter period for full conversion to ethanol. Ethanol production profile for the experiment with 5.0% (w/v) sago hampas increased rapidly from 0 to 12 h (at 72.22% TEY) and remained at the range of 76.23% to 77.66% TEY. At 48 h, ethanol production increased slightly to 79.65% TEY but decreased to 63.06% TEY at 120 h. Additionally, the ethanol yield at 5.0% (w/v) sago hampas loading in the present study was favourably comparable to those described in other previous studies (Kannan et al. 2013; Thangavelu et al. 2014; Vincent et al. 2015a, b).

Figure 6 shows the acetic acid production profiles. At 24 h, acetic acid production in broth with 2.5% (w/v) sago hampas peaked at 1.49 g/L, and, remained virtually constant until the end of the SSF period. Meanwhile, in the broth with 5.0% and 7.5% (w/v) sago hampas, acetic acid increased steadily until 120 h to final concentrations of 1.85 g/L and 2.07 g/L, respectively. One possible explanation for the increment in the rate of acetic acid production is the biological oxidation of ethanol. Additionally, the presence of acetic acid is also a vital indicator to gauge biomass hydrolysis, as the increase in acetic acid levels indicates continuous degradation of lignocellulosic materials during SSF (Vincent et al. 2015a).

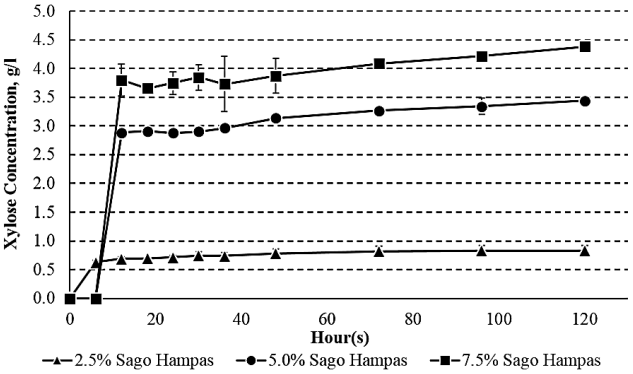

Figure 4. Time course of xylose profile during SSF, as determined via HPLC

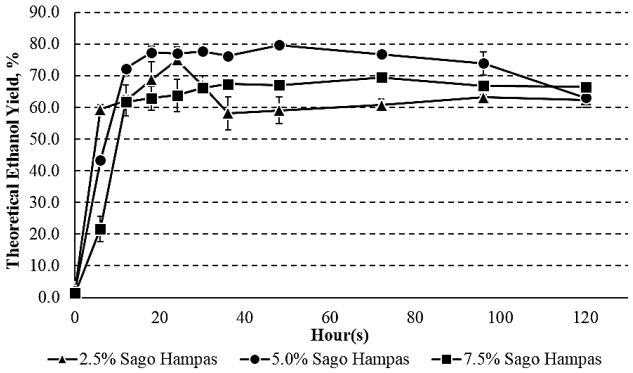

Figure 5. Time course of theoretical ethanol yield (TEY) profile during SSF

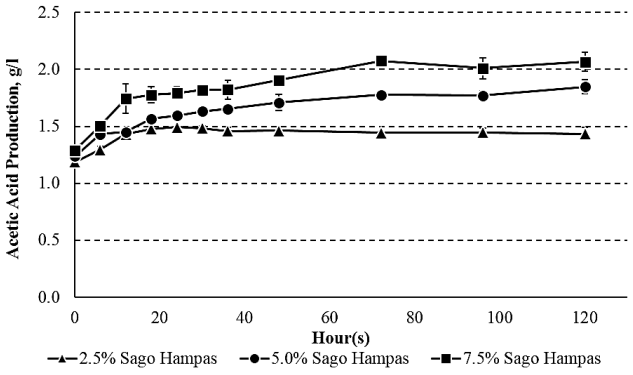

Figure 6. Time course of acetic acid profile during SSF, as determined via HPLC

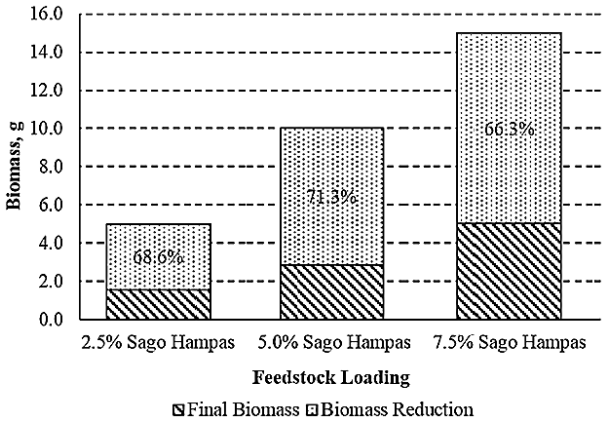

Figure 7. Biomass reduction of sago hampas after the SSF process

Determination of biomass reduction

By the end of the SSF period, total biomass reduction was recorded between 66.3% to 71.3% (Figure 7), indicating that less than 35.0% of the original substrate remained unutilised. These observations are encouraging as this study demonstrated that bioethanol production using fresh sago hampas may assist in reducing the environmental burden from the sago waste released by starch producing industries. Eventually, this may alleviate the needs for alternative waste management (Linggang et al. 2012; Kannan et al. 2013).

In conclusion, fresh sago hampas is shown to be a potential bioethanol feedstock when subjected to simultaneous saccharification and fermentation (SSF) with the aid of amylolytic-cellulolytic enzymes and S. cerevisiae. The results showed that SSF with 5.0% (w/v) sago hampas loading was the most effective in generating highest ethanol yield at 79.65% TEY.

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