Sago Biomass as a Sustainable Source for Biohydrogen Production by *Clostridium butyricum* A1

Mohd Azwan Jenol,[a] Mohamad Faizal Ibrahim,[a] Phang Lai Yee,[a] Madihah Md Salleh,[b] and Suraini Abd-Aziz [a],*

Biohydrogen production from biomass is attracting many researchers in developing a renewable, clean and environmental friendly biofuel. The biohydrogen producer, *Clostridium butyricum* A1, was successfully isolated from landfill soil. This strain produced a biohydrogen yield of 1.90 mol H_2/mol glucose with productivity of 170 mL/L/h using pure glucose as substrate. The highest cumulative biohydrogen collected after 24 h of fermentation was 2468 mL/L-medium. Biohydrogen fermentation using sago *hampas* hydrolysate produced higher biohydrogen yield (2.65 mol H_2/mol glucose) than sago pith residue (SPR) hydrolysate that produced 2.23 mol H_2/mol glucose. A higher biohydrogen productivity of 1757 mL/L/h was obtained when using sago *hampas* hydrolysate compared to when using pure glucose that has the productivity of 170 mL/L/h. A comparable biohydrogen production was also obtained by *C. butyricum* A1 when compared to *C. butyricum* EB6 that produced a biohydrogen yield of 2.50 mol H_2/mol glucose using sago *hampas* hydrolysate as substrate. This study shows that the new isolate *C. butyricum* A1 together with the use of sago biomass as substrate is a promising technology for future biohydrogen production.

Keywords: Biohydrogen; Sago hampas; Sago pith residue; Fermentable sugars; Clostridium butyricum

*Contact information: a: Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; b: Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia; *Corresponding author: suraini@upm.edu.my*

INTRODUCTION

Biohydrogen gas produced through fermentation processing is an ideal alternative fuel as a sustainable and clean bioenergy carrier for the future. Biohydrogen is a simple gas with a molecular formula of H_2 and has a high yield of energy (122 kJ/g), which is 2.75 times higher compared to fuels derived from petroleum (Pattra *et al.* 2008; Chong *et al.* 2009; Claassen *et al.* 2010). The combustion of hydrogen is better compared to petroleum-derived fuels, since the only byproduct generated is water (Fields 2003; Pattra *et al.* 2008). Thus, the utilization of hydrogen as a fuel subsequently reduces the release of carbon dioxide into the atmosphere. At present, the industrial production of hydrogen is carried out via several processing routes including steam reforming of methane, electrolysis of water, and thermochemical reaction (Steinfeld 2005; Levin and Chahine 2010). The major problems among these technologies are cost-ineffectiveness and high energy consumption. Therefore, innovative and novel biohydrogen production via fermentation has now been attempted due to the needs of minimizing the amount of land required for the operation, and making the system insensitive to weather conditions (Ni *et al.* 2006). In European countries, the hyvolution concept has been introduced by

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exploiting bacteria that are capable in producing biohydrogen as a byproduct during growth on biomass (Claassen *et al.* 2010). Besides, the utilization of biomass as a fermentation feedstock can eliminate the cost of raw materials, which typically contributes around 50 to 70% of the total biohydrogen production cost. This approach will also help biomass-related industries in managing their biomass waste and to generate extra profit (Hassan *et al.* 2004).

The sago biomass considered in this work is composed of mainly starch. The polysaccharides of starch, cellulose, and hemicellulose can be hydrolyzed into simple sugars, which can be utilized as a carbon source for the fermentation process by microorganisms. The bioconversion of biomass into simple sugars can be conducted through the hydrolysis process using specific enzymes. Several types of biomass have been used, including oil palm empty fruit bunch (Inayat *et al.* 2012), palm oil mill effluent (Chong *et al.* 2009), cassava wastewater (Sreethawong *et al.* 2010), and sweet potato starch residue (Yokoi *et al.* 2001) for the production of biohydrogen.

Malaysia is blessed with a favorable climate and abundant natural resources for commercial crop cultivation such as sago palm. The Malaysian sago palm industry has been accounted as one of the most important sago exporters in the world and exports are more than 25,000 mt of sago starch every year (Senic 2002). The Department of Agriculture Sarawak (DAS) (2010) has reported that the export value has increased by 15 to 20% per year. The upward trend in sago production will significantly increase the amount of waste generated from this industry, which may cause waste management problems and contribute to environmental pollution. This industry has generated a vast amount of waste in the form of by-products including sago bark, sago *hampas*, and sago wastewater. Sago *hampas* contains (on a dry weight basis) 58% starch, 23% cellulose, 9.2% hemicellulose, and 3.9% lignin (Ozawa *et al.* 1996; Awg-Adeni *et al.* 2012; Linggang *et al.* 2012). The hydrolysis of starch material in sago *hampas* into glucose produces another by-product which is sago pith residue (SPR). Linggang *et al.* (2012) had reported that SPR is composed of (on a dry weight basis) 37% cellulose, 20% hemicellulose, and 6% lignin; the cellulosic materials can be hydrolyzed into a mixture of fermentable sugars using cellulase enzymes. These types of biomass have the potential to be utilized as a carbon source in the production of valuable products. Thus, a study of the utilization of sago *hampas* and SPR for biohydrogen production was conducted. A new local isolated strain *Clostridium butyricum* A1 was employed to conduct the biohydrogen fermentation process to be compared with a well-known biohydrogen producer, *Clostridium butyricum* EB6 isolated by Chong *et al.* (2009).

A variety of biohydrogen-producing microorganisms has been documented by several studies. To date, *Clostridium* sp. is widely employed for biohydrogen production, since this species has the ability to convert hexose sugar to biohydrogen with a theoretical yield of 4 mol H_2/mol hexose. The theoretical yield is higher as compared to the biohydrogen produced by *Enterobacter* sp., which is 1 mol H_2/mol hexose (Kotay and Das 2008). Besides, several studies have also been done on *Enterobacter* sp., since this species can achieve a higher production rate than other reported species (Chen *et al.* 2005; Kotay and Das 2008; Prasertsan *et al.* 2009). However, *Clostridium* sp. has the capability to produce a biohydrogen yield in the range of 1.4 to 2.8 mol H_2/mol glucose (Chen *et al.* 2005; Lin and Tanaka 2006; Levin and Chahine 2010). The highest biohydrogen yield that has been reported was 3.26 mol H_2/mol glucose by employing *C. butyricum* (Keskin and Hallenbeck 2012). There are also several reports on the production of biohydrogen by *Thermotoga* sp. using biomass as substrate with a yield in

the range of 1.1 to 2.0 mol H_2/mol glucose or other hexoses (Evvyernie *et al.* 2001; Mars *et al.* 2010).

This study was conducted to investigate the ability of locally isolated biohydrogen producer, *C. butyricum* A1 for the production of biohydrogen. Hence, this research also aimed to investigate the feasibility of sago biomass in the two forms of sago *hampas* and sago pith residue on the biohydrogen production by *C. butyricum* A1.

EXPERIMENTAL

Experimental Design

Sago *hampas* collected from sago industry was used as raw material for biohydrogen production. This sago *hampas* was hydrolyzed by dextrozyme into sago *hampas* hydrolysate, while the remaining solid residue was collected as sago pith residue after it was separated using a centrifuge. The solid sago pith residue was subjected for enzymatic hydrolysis by crude cellulase produced by *Aspergillus fumigatus* UPM2, into fermentable sugars called sago pith residue hydrolysate. Both hydrolysates (sago *hampas* and sago pith residue) were used as carbon source for biohydrogen production by new isolated strain (*Clostridium butyricum* A1) and by a known biohydrogen producer (*Clostridium butyricum* EB6). The overall experimental design is shown in Fig. 1.
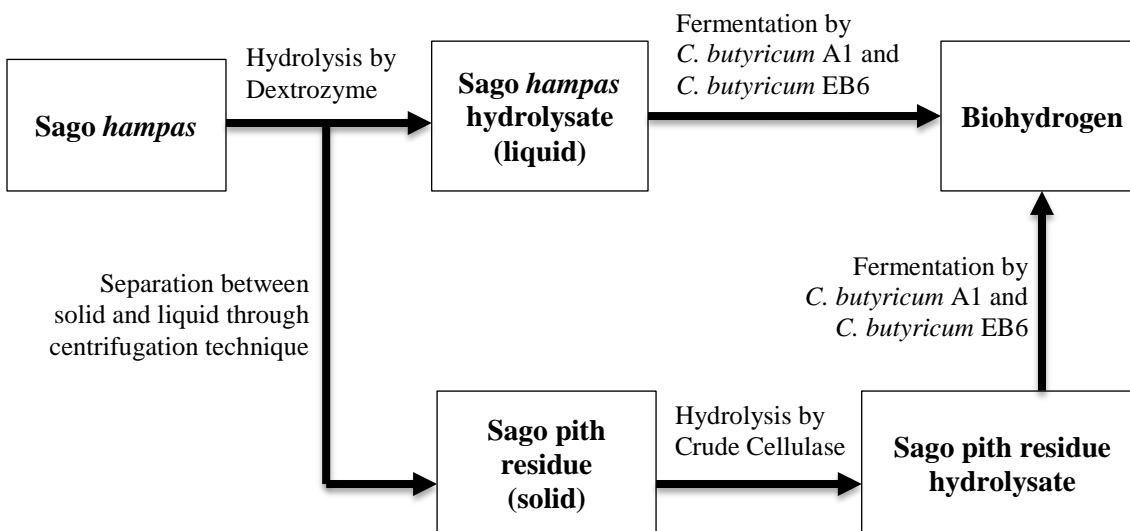

Fig. 1. Overall experimental design for biohydrogen production from sago biomass

Screening, Isolation, and Identification of Biohydrogen Producing Bacteria
Screening and isolation

The biohydrogen producing bacteria were successfully isolated from a soil sample collected from Bukit Tagar Landfill, Selangor, Malaysia by Zamzuri (2010). A 1 g specimen of landfill soil was dispersed in 10 mL of saline water, and then 1 mL of suspension was cultured on the nutrient agar and incubated in an incubator at 37 °C for 24 h. After that, the screening process was done by subculturing the bacteria on reinforced *Clostridia* medium (RCM) agar. The RCM agar was prepared by dissolving 38 g of ready-made RCM (Merck, Denmark) and 13 g of bacteriological agar (BD, France) in 1000 mL of distilled water before it was autoclaved at 121 °C for 15 min. This screening process was repeated until a single colony was obtained. The single colony was

isolated and transferred into RCM broth aseptically and anaerobically using an anaerobic chamber (Bactron[TM], Sheldon, USA). The bacterium named as strain A1 was successfully isolated and employed for biohydrogen production. This isolated strain was cultured in RCM broth for 24 h in a shaker incubator (Labwit, China) at 37 °C with 120 rpm of agitation speed prior to the biohydrogen fermentation. The RCM broth was prepared by dissolving 38 g of ready-made RCM (Merck, Denmark) in 1000 mL of distilled water. Then, 100 mL of RCM broth was transferred into 125 mL of serum bottle and sparged with nitrogen gas for 15 min before it was autoclaved at 121 °C for 15 min.

DNA extraction

The high yield gel/PCR DNA fragment extraction kit (Real Genomics[TM], Taiwan) was used for DNA extraction. A 1 mL of culture was withdrawn from stock culture and centrifuged at 5000 rpm, 4 °C for 5 min using a bench top centrifuge (5415D, Eppendorf, Germany). The pellet was retained and washed with sterile distilled water for twice. The DNA was extracted according to the manufacturer's instructions.

PCR amplification, sequencing, and phylogenetic analysis

The PCR amplification, sequencing and phylogenetic analysis were done based on the study by Chong *et al.* (2009). The PCR amplification was conducted using the Takara Ex Taq[TM] kit. The 16s rRNA gene was amplified with two sets of universal primers. The two primers were, respectively, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR amplification product was purified using the QIAgen gel extraction kit according to manufacturer's instructions prior to sequence analysis. The phylogenetic trees were constructed using the neighbor-joining method using MEGA 5.05 software.

Substrate Preparation

Sago waste was collected from Herdsen Sago Industries Sdn. Bhd., Pusa' in Sarawak, Malaysia. The wet sago *hampas* was packed in porous bags in order to allow the supernatant to be drained off naturally for 1 to 2 days. Further, it was dried overnight using oven at 65 °C. The dried sago *hampas* was then stored at room temperature in a sealed plastic bag prior to the saccharification process.

Cellulase Production

The production of crude cellulase was conducted using sago pith residue (SPR) (which was obtained after saccharification of sago *hampas* by dextrozyme) by *Aspergillus fumigatus* UPM2 based on the study by Linggang *et al.* (2012). *A. fumigatus* UPM2 was cultured on potato dextrose agar (PDA) and incubated in an incubator (Memmert, Germany) for 7 days at 30 °C. Then, 10 mL of sterilized 0.1% (v/v) of Tween 80 was used to harvest the spore suspension. The surface of the cultured PDA was rubbed gently using a sterilized hockey stick and transferred into a sterilized universal bottle prior to fermentation.

The fermentation was conducted using 1% (w/v) of SPR in a 250 mL conical flask that was autoclaved at 121 °C for 15 min. A 100 mL of Mendel media containing (in g/L): KH_2PO_4, 0.2; $CaCl.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; $MnSO_4.H_2O$, 1.6; $ZnSO_4.H_2O$, 5.0; $CoCl_2$, 2.0; Tween 80, 2.0 mL; Trace element, 1.0 mL was added into the conical flask. The prepared medium was inoculated with $1x10^6$ spores/mL of *A.*

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fumigatus UPM2. The inoculated medium was then incubated using a shaker incubator (Labwit, China) for 7 days at 30 °C, 150 rpm.

The crude cellulase was harvested using the centrifugation and filtration technique, and stored at 4 °C for subsequent use. The cellulase activity of crude cellulase produced was analyzed based on the methods by Wood and Bhat (1988).

Saccharification

Sago hampas

Sago *hampas* composed of 49% (based on a dried basis) starch was used in the hydrolysis process to produce glucose based on the method by Awg-Adeni *et al.* (2012). A 7% (w/v) of sago *hampas* was weighed in 0.1 M of phosphate buffer solution at pH 4. Then, it was boiled to gelatinize it for 15 min before it was cooled down to 60 °C at room temperature. A 5.56 mL of dextrozyme (Novozymes, Denmark) with glucoamylase activity of 195.3 U/mL was added into the flask. The mixture was incubated in a water bath at 60 °C for 60 min for the hydrolysis process. The mixture was stirred to homogenize the substrate and the enzyme throughout the process.

The sugars produced from the hydrolysis were recovered using a centrifuge (Multifuge x3R, Thermo Scientific Heraeus, Germany) at 4 °C and 4000 rpm followed by filtration using 1.0 µm membrane filter paper attached to a vacuum pump. The hydrolysis yield was calculated based on Eq. 1 (Awg-Adeni *et al.* 2012). The recovered sugar, namely sago *hampas* hydrolysate, analyzed the sugar content and was stored at 4 °C prior to biohydrogen fermentation. The solid residue containing lignocellulosic materials produced from the hydrolysis process, known as SPR was collected and subjected to further saccharification.

$$\text{Hydrolysis yield} = \frac{\text{Glucose recovered from starch of sago hampas (g)}}{\text{Dried sago 'hampas' (g)}} \times 100 \quad (1)$$

Sago pith residue

The saccharification of SPR was carried out using crude cellulase produced as in "Cellulase production" following the method as described by Linggang *et al.* (2012). Enzymatic hydrolysis of SPR was performed using 5% (w/v) of SPR loaded in 100 mL of 0.05 M acetate buffer at pH 4.8. The process was carried out for 3 days in a shaker incubator (Labwit, China) at 50 °C and 200 rpm. The SPR hydrolysate produced from this hydrolysis was recovered by centrifuging at 3500 rpm for 10 min using a centrifuge (Multifuge x3R, Thermo Scientific Heraeus, Germany) followed by a filtration process using 1.0 µm pore size of membrane filter. The recovered SPR hydrolysate was stored at 4 °C prior to the biohydrogen production process. The hydrolysate was analyzed to determine the reducing sugar and glucose concentrations. The hydrolysis percentage was calculated using Eq. 2 (Linggang *et al.* 2012).

$$\text{Hydrolysis (\%)} = \frac{[\text{Total reducing sugar (g/L)} \times 0.9 \times 100]}{\text{Substrate (g/L)} \times [\text{hemicellulose+cellulose (g/g)}]} \quad (2)$$

The correction factor of 0.9 was included in the calculation, since the hydrolysis of polysaccharides involves water, where for every 1 mole of reducing sugar released, 1 mole of water is required.

Biohydrogen Production

Inoculum preparation

   C. butyricum A1 and *C. butyricum* EB6 were employed for biohydrogen production. A 1 mL aliquot of stock culture for both strains was cultured in 100 mL of oxygen free and sterilized RCM broth, pH 5.5. Then, it was incubated in a shaker incubator (Labwit, China) at 37 °C and 120 rpm for 24 h prior to the biohydrogen fermentation.

Medium preparation

   The basal medium was prepared according to Chong *et al.* (2009). The carbon source, which is glucose, was 10 g/L. The pH of the medium was set at pH 6.5 using 1.0 M of NaOH. The carbon source was replaced with sago *hampas* hydrolysate and SPR hydrolysate based on the glucose concentration in the hydrolysate. The prepared medium was transferred into a 125 mL serum bottle and sparged with nitrogen for 15 min before it was autoclaved at 115 °C for 5 min.

Fermentation

   A 10% (v/v) of culture was aseptically transferred into the basal medium and incubated using a shaker incubator (Labwit, China) at 37 °C for 24 h. A 2 mL of sample was collected at 3-h intervals. The gas produced was collected using syringes and kept in a Hungate tube prior to analysis. The fermentation was repeated three times to obtain average results.

Analytical Procedures

   Cellulose, hemicellulose, and lignin composition of sago *hampas* and SPR were determined based on the method by Goering and Van Soest (1970). The cellulase activities (β-glucosidase, CMCase, and FPase) were analyzed using the methods by Wood and Bhat (1988). The reducing sugar concentration was analyzed using the dinitrosalicylic acid (DNS) method as reported by Miller (1959), and the glucose concentration was analyzed using High Performance Liquid Chromatography (HPLC), following the methods by Linggang *et al.* (2012). The hydrogen concentration was analyzed using gas chromatography (GC-A8, Shimadzu, Japan) with a thermal conductivity detector. The gas carrier used was nitrogen gas and the column was packed with Porapak Q (80/100 mesh). The temperatures at the stainless column and the injection and detector point was set at 50 °C, 50 °C, and 100 °C, respectively. The standard hydrogen gas was used to plot the hydrogen production curve (Chong *et al.* 2009).

Kinetic Modeling

   In batch biohydrogen fermentation, the cumulative biohydrogen produced was determined based on the modified Gompertz equation,

$$H = P \exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \tag{3}$$

where *H* is cumulative hydrogen production (mL), *P* is the hydrogen production potential (mL H_2), *λ* is the lag phase time (h), *t* is the incubation time (h), R_m is the maximum hydrogen production rate (mL H_2/h), and *e* has a value of 2.718281828. The values of *P*,

λ, and R_m were estimated using a non-linear estimation function in STATISTICA (version 8.0). The biohydrogen yield was determined based on the total accumulated hydrogen produced, divided with the amount of glucose consumed.

RESULTS AND DISCUSSION

Chemical Composition of Sago *Hampas* and Sago Pith Residue

The chemical compositions of sago *hampas* and SPR are shown in Table 1. The starch content in the sago *hampas* was 49.5% (on a dry weight basis), which was almost comparable to the starch contents reported by previous studies, which were in the range of 30 to 50% (Barrett 2008; Awg-Adeni *et al*. 2010). During the saccharification of sago *hampas* by dextrozyme, the enzyme only attacked the starch content in the sago *hampas* to be converted into glucose, while the lignocellulosic materials were left as SPR. This SPR contained 58.5% of potential sugar (cellulose + hemicellulose), which was comparable to the findings of Linggang *et al.* (2012). These cellulose and hemicellulose polysaccharides are made of complex glucose monomer units linked by the 1-4-β-glycosidic bond (Rubin 2008). These polysaccharides can be used to induce the celluloytic microorganism to produce crude cellulase, as have been done by Linggang *et al.* (2012), Razak *et al.* (2012), and Ibrahim *et al.* (2013). The cellulase produced can subsequently be used to hydrolyze SPR into its monomers, mainly glucose, xylose, mannose, and arabinose (Linggang *et al.* 2012).

Table 1. Chemical Composition of Sago *Hampas* and Sago Pith Residue on Dry Weight Basis

Chemical composition (%)	Sago *hampas*	Sago pith residue
Starch	49.5	nd
Cellulose	26.0	44.0
Hemicellulose	14.5	14.5
Lignin	7.5	4.9
Others	2.5	4.7

nd – not detected

Saccharification of Sago *Hampas* and Sago Pith Residue

The saccharification of sago *hampas* is summarized in Table 2. The saccharification of sago hampas produced 28.5 g/L of reducing sugars, which represented 40.8% of hydrolysis yield (Table 2). It should be noted that the percentage composition of starch in sago *hampas* was 49.5%, and the remaining unhydrolyzed starch was 8.7%. The hydrolyzed starch from dextrozyme hydrolysis produced 94.3% glucose, 2.4% cellobiose, 1.8% maltose, and 1.5% of other sugars, including fructose and mannose as revealed by analysis using HPLC.

Lower pH was set in the saccharification of sago *hampas* in order to ensure better hydrolysis of the glycosidic bond present in starch structure (van der Maarel *et al.* 2002). The starch granules were disrupted during the gelatinization process at boiling temperature aimed to increase the activity of enzyme to hydrolyze the starch (Reeve 1992; Awg-Adeni *et al*. 2012).

Table 2. Saccharification of Sago *Hampas* and Sago Pith Residue

Substrate	Substrate loading (% w/v)	Reducing sugar (g/L)	Glucose concentration (g/L)	Hydrolysis percentage (%)	References
Sago *hampas*	7	31.1	27.8	40	Awg-Adeni *et al.* (2012)
		30.2	28.5	41	This study
Sago pith residue	5	20.8	Na	73	Linggang *et al.* (2012)
		20.7	12.4	71	This study

na = not available

The solid residue after the saccharification of sago *hampas* is SPR, a lignocellulosic-based biomass. Thus, the saccharification of SPR was conducted using cellulase, an enzyme that hydrolyze the 1,4-β-glycosidic bonds of the cellulose and hemicellulose structure into a mixture of fermentable sugars. This cellulase was produced from *A. fumigatus* UPM2, as described in the "Material and Methods". An initial β-glucosidase activity of 10 U/mL was used based on the data reported by Linggang *et al.* (2012). β-glucosidase was chosen as the cellulase indicator because it has been well accepted as the rate limiting enzyme in the hydrolysis of cellulosic material (Kadam and Demain 1989). This hydrolysis of SPR produced 20.73 g/L of total fermentable sugars with the hydrolysis percentage of 71.4%. Analysis using HPLC showed that 12.8 g/L of glucose was detected, followed by 2.6, 2.4, 1.7, and 1.2 g/L of maltose, arabinose, mannitol, and cellobiose, respectively. Cellulose is composed of glucose monomers, while hemicellulose is made of monomers of pentoses and hexoses (glucose, arabinose, xylose, and mannose) (Festucci-Buselli *et al.* 2007). Saccharification of this cellulosic material involves multi-domain proteins consisting of three major cellulase components, which are endo-β-(1,4)-D-glucanase (CMCase), exo-β-(1,4)-D-glucanase (FPase), and β-glucosidase. These cellulase enzyme components synergistically attack the complex cellulose and hemicellulose structures (Reeve 1992). In this process, initially, endo-β-(1,4)-D-glucanase randomly cleaves the β-(1,4)-linkages of the glucose chain in the amorphous component of the cellulose to loosen and opens up the cellulose structure to allow it to be attacked by exo-β-(1,4)-D-glucanase (Esteghlalian *et al.* 2002). The exo-β-(1,4)-D-glucanase, also known as cellobiohydrolase, acts to release the cellobiose moiety from the end of the oligosaccharide chain. Finally, β-glucosidase acts to release the glucose from cellobiose and from the short chain of cello-oligosaccharides (Krishna *et al.* 1998; Rajoka *et al.* 2004; Ikram-ul-Haq *et al.* 2005). The whole process produces a mixture of fermentable sugars.

The sugar hydrolysate produced from the saccharification of sago *hampas* and SPR was used as substrate for biohydrogen production by a new local isolate *C. butyricum* A1 and by a known biohydrogen producer, *C. butyricum* EB6.

Identification and Phylogenetic Analysis of Strain A1

Strain A1 isolated by Zamzuri (2010) was characterized as a Gram negative bacterium and appeared as a single short rod under 100x magnification using a light microscope. The phylogenetic analysis (Fig. 2) shows that strain A1 has higher similarity towards the *Clostridium butyricum* RCEB (EU621841.1) with maximum similarity percentage of 99.9% when compared with the sequences available in the NCBI database. Strain A1 clustered with the known *Clostridium* sp. and fell in the species cluster of *C. butyricum*. *C. butyricum* is a strictly anaerobic bacterium (Chong *et al.* 2009; Claassen *et al.* 2010) and has been employed in several studies for biohydrogen production (Fang *et al.* 2002; Lin and Tanaka 2006; Chong *et al.* 2009), as well as for butyric acid production (Wei *et al.* 2013). Yokoi *et al.* (1998) mentioned that *C. butyricum* is one of the effective biohydrogen producers. A study by Chong *et al.* (2009) also reported that their local isolate *C. butyricum* EB6 produced a high yield of biohydrogen when compared to other isolates. Thus, in this study, the ability of the new locally isolated *C. butyricum* A1 was investigated and compared with *C. butyricum* EB6, using synthetic glucose, sago *hampas* hydrolysate, and SPR hydrolysate as substrate.

Biohydrogen Production using Different Types of Carbon Source

Glucose

C. butyricum is a strictly anaerobic bacterium that thrives in the absence of oxygen. The biohydrogen production by *C. butyricum* A1 was conducted and compared with the well known biohydrogen producer *C. butyricum* EB6, as reported by a previous study (Chong *et al.* 2009). Figure 3 shows the biohydrogen production profile by both strains in terms of cell growth and glucose consumption (Fig. 3a), pH changes (Fig. 3b), cumulative biogas and biohydrogen production (Fig. 3c), and biohydrogen production rate (Fig. 3d). The biohydrogen percentage detected in this study was in the range of 60 to 70%. Based on the results obtained, *C. butyricum* A1 produced 246.8 NmL of total cumulative biohydrogen gas with 9.7 g/L of total glucose consumed out of 10.0 g/L of glucose supplemented in the fermentation. This biohydrogen production was slightly higher when compared to the total cumulative biohydrogen produced by *C. butyricum* EB6, which was 232.5 NmL with 9.7 g/L of glucose consumed. *C. butyricum* A1 also gave a comparable biohydrogen yield (1.90 mol H_2/mol glucose) when compared to *C. butyricum* EB6 that produced 1.79 mol H_2/mol glucose.

During this fermentation, the modified Gompertz equation (Eq. 3) was used to estimate the hydrogen production potential (in mL), maximum hydrogen production rate (in mL/L), and lag phase (in h) using the STATISTICA (version 8.0) software. As a result, the biohydrogen production potential by *C. butyricum* A1 was significantly higher when compared to *C. butyricum* EB6, equivalent to 260.7 mL and 245.6 mL, respectively. Besides, the maximum hydrogen production rate obtained by Chong *et al.* (2009) was 172.0 mL/L/h, which was almost comparable to *C. butyricum* A1 that had produced 170.0 mL/L/h. Therefore, it is suggested that the local isolate *C. butyricum* A1 has the potential to be employed to produce biohydrogen.

As illustrated in Figs. 3(a) and 3(c), the cells and biohydrogen were rapidly increasing at 6 h and entered the stationary phase at 18 h of incubation time. This situation indicates that the biohydrogen is a primary metabolite that is associated with the cell growth (Chong *et al.* 2009). Based on the following reviews (Grupe and Gottschalk 1986; Terracciano and Kashket 1986; Jones and Woods 1986), excess proton was emitted

during the primary metabolism of *C. butyricum* and reduced to form molecular hydrogen to be disposed of in order to maintain the electrical neutrality of the system.
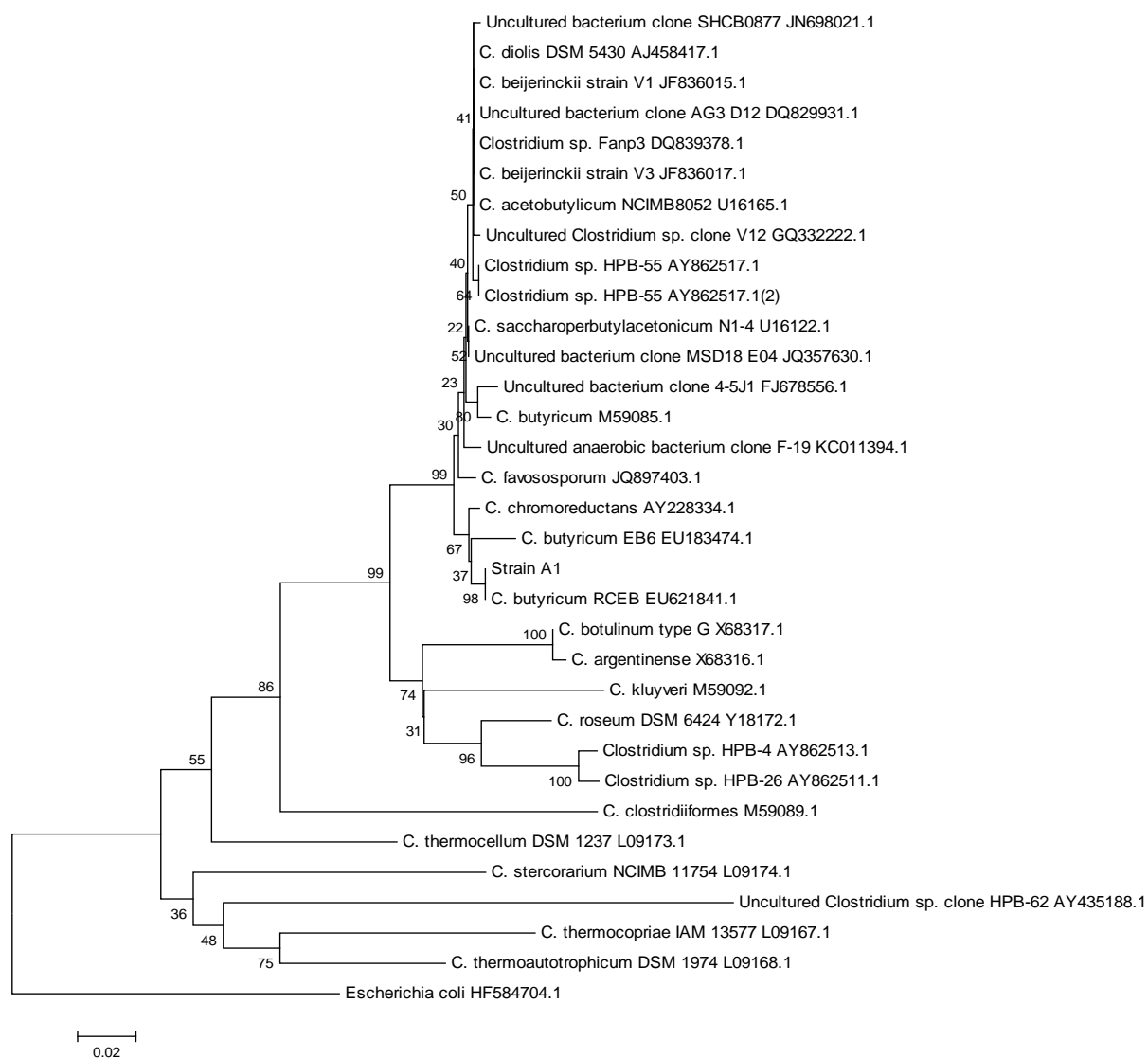

Fig. 2. The phylogenetic tree of the anaerobic biohydrogen-producing strain based on the 16s rDNA sequence and constructed according to Jukes-Cantor distance using a neighbor-joining method with 1000 bootstrappings. *Escherichia coli* was chosen as the outgroup species. The bootstrap values are indicated at the nodes with the scale bar represents 0.02 substitutions per nucleotide position. Reference sequences in the tree were obtained from NCBI with their accession numbers.

Hydrogen started to be produced right after the inoculation, and the maximum production occurred when the cell growth was near to the stationary phase. The highest biohydrogen production rate was found within 9 h after fermentation with the rate of 35.15 mL/h for *C. butyricum* A1 and 43.63 mL/h for *C. butyricum* EB6. The lag phase was 8.2 h for *C. butyricum* A1, while for *C. butyricum* EB6, it was 9.8 h which was almost comparable to the results obtained by Nazlina *et al.* (2009). The glucose concentration was consumed throughout the fermentation due to the cell replication and product formation. Based on Figure 3(b), the pH decreased drastically when hydrogen

production started. This indicated that the *C. butyricum* was undergoing acidogenesis. It should be noted that *Clostridium* sp. undergoes two phases of fermentation, acidogenesis and solventogenesis. According to Ramey and Shang (2004), during acidogenesis, acids such as butyric acid, acetic acid, and lactic acid are produced together with hydrogen and carbon dioxide as byproducts, while during solventogenesis, solvents, such as acetone, butanol, and ethanol are produced.
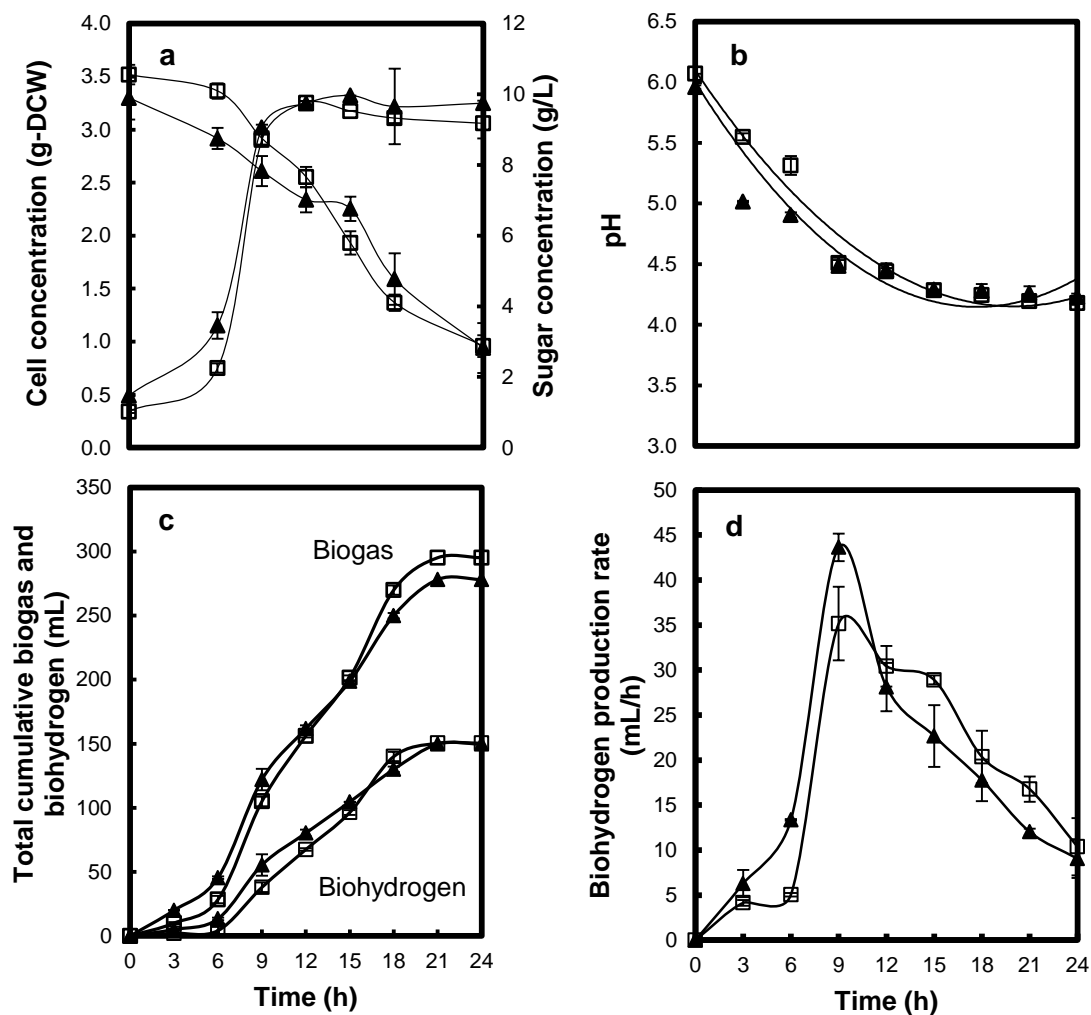

Fig. 3. Biohydrogen production using 10 g/L of glucose by *Clostridium butyricum* A1 (□) and *Clostridium butyricum* EB6 (▲). Profile of (a) cell growth and glucose consumption, (b) pH changes, (c) cumulative biogas and biohydrogen production, and (d) biohydrogen production rate.

The biohydrogen production and cell growth decreased at 18 h due to the accumulation of certain acids when the cells entered the stationary phase (Jones and Woods 1986; Ginkel and Logan 2005). At this stage, the cells were penetrated by nonpolar undissociated acids, and higher intracellular pH was achieved, which later became toxic to the cell. Acid inhibition in *Clostridium* sp. had been reported by several researchers (Maddox *et al.* 2000; Saratale *et al.* 2008; Ibrahim *et al.* 2012) when acid accumulation above the threshold value was detected just before the cells began to enter the stationary phase.

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Sago *Hampas* Hydrolysate

The biohydrogen production was conducted using 10.0 g/L of glucose from sago *hampas* by *C. butyricum* A1 and *C. butyricum* EB6. Figure 4 shows the profiling of cumulative biohydrogen production from sago hampas by *C. butyricum* A1 and *C. butyricum* EB6. A comparable biohydrogen production was obtained from both strains, with cumulative biohydrogen equivalent to 394 mL for *C. butyricum* A1 and 388 mL for *C. butyricum* EB6. Both strains produced 2.65 and 2.50 mol H_2/mol glucose with a productivity of 1757 and 1720 mL/L/h for both *C. butyricum* A1 and *C. butyricum* EB6, respectively, as shown in Table 3. There were almost the same results obtained in both strains, showing that the newly isolated *C. butyricum* A1 has the same potential as *C. butyricum* EB6. However, the lag phase shown by *C. butyricum* EB6 (2 h) was significantly shorter compared to *C. butyricum* A1, which had a lag phase of 5 h. This situation might be due to the adaptability of the strain to produce acids in the first place. At 3 h of fermentation, the pH value in the fermentation by *C. butyricum* EB6 showed a rapid drop from 6.5 to 5.5, indicating that the cell started to enter acidogenesis, while for *C. butyricum* A1, the pH of the system decreased after 6 h of fermentation. The biohydrogen production was dependent on acetate, propionate, and butyrate (Berkers *et al.* 2010) derived from an acidogenic phase of *Clostridia*. Thus, it is suggested that the faster the acid production, the faster the biohydrogen will be produced. However, the level of the acids produced must not be too high, as they will inhibit the hydrogen generation. In anaerobic fermentation by *Clostridia*, the hydrogenase (the enzyme responsible for hydrogen production) oxidizes the ferrodoxin into molecules of hydrogen. The addition of iron in the culture medium may enhance the activity of the hydrogenase. However, the hydrogenase associated with the iron element would be affected by the low pH level, at the stage where high concentration of acid was produced (Dabrock *et al.* 1992).

The biohydrogen production using sago *hampas* hydrolysate produced 1.6-fold higher cumulative biohydrogen and 10-fold faster production rate when compared to biohydrogen production using synthetic glucose. The significantly higher cumulative biohydrogen level obtained might be due to the presence of 8% of the unhydrolyzed starch in the sago *hampas* hydrolysate, where *C. butyricum* A1 might also convert the remaining starch in the hydrolysate to biohydrogen, thus giving a higher cumulative biohydrogen production. The presence of other sugars released after the saccharification of sago *hampas* might also enhance the biohydrogen production. This situation was demonstrated by Berkers *et al.* (2010), where pure culture of *C. butyricum* successfully produced higher biohydrogen production from starch compared to synthetic glucose.
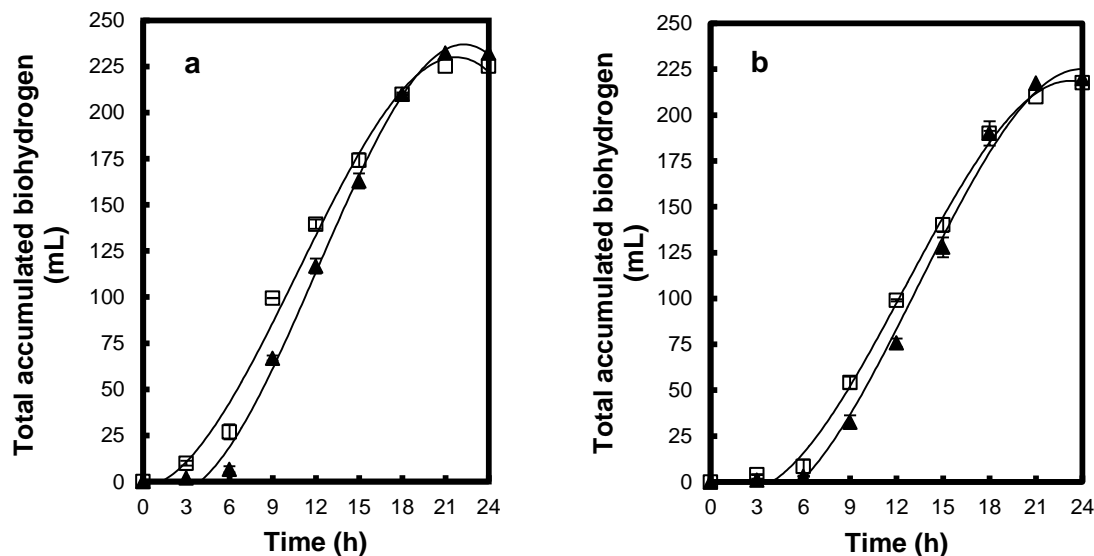
Fig. 4. Biohydrogen production from sago *hampas* hydrolysate (a) and from sago pith residue (b) by *Clostridium butyricum* A1 (▲) and *Clostridium butyricum* EB6 (□)

Sago Pith Residue Hydrolysate

Biohydrogen production using SPR hydrolysate by *C. butyricum* A1 and *C. butyricum* EB6 was also conducted. The profiling of cumulative biohydrogen produced is shown in Fig. 4b. Similar to the fermentation using sago *hampas* hydrolysate, these two strains showed a similar biohydrogen fermentation behavior. The modified Gompertz equation predicted that *C. butyricum* EB6 would produce a cumulative biohydrogen of 332.2 mL with a productivity of 1578.1 mL/L/h, while *C. butyricum* A1 would produce 304.8 mL and 1643.1 mL/L/h of cumulative biohydrogen and productivity, respectively, as presented in Table 3. In comparison to the biohydrogen production using synthetic glucose, higher cumulative biohydrogen and productivity were obtained when using SPR hydrolysate as substrate, which were 1.2-fold higher in terms of cumulative hydrogen and 9.7-fold higher for maximum productivity.

The situation just described indicated that SPR hydrolysate is a superior carbon source compared to pure glucose. In the biohydrogen fermentation using pure glucose, the pH of the system was reduced to 4.1, while in the biohydrogen fermentation using SPR hydrolysate, the pH dropped to 4.8. The decrease in the pH value was attributable to the formation of acids (acetate and butyrate), and this was the main factor in inducing biohydrogen production. It is well accepted that pH is the most important parameter in biohydrogen fermentation, since the pathways involved in *Clostridia* are influenced by the amount of acid as indicated by the acidic pH value (Berkers *et al.* 2010). However, in comparison to the fermentation using sago *hampas* hydrolysate, the fermentation using SPR hydrolysate produced a slightly lower cumulative biohydrogen amount and lower productivity for both *C. butyricum* A1 and *C. butyricum* EB6. The increment in the total accumulated biohydrogen production and the maximum hydrogen productivity in sago *hampas* as compared to SPR hydrolysate were 1.3-fold and 1.1-fold, respectively. A slightly longer lag phase (7 h) also happened in this fermentation, which indicated the disadvantage of SPR hydrolysate over sago *hampas* hydrolysate. This situation might be due to the presence of saccharides in the SPR hydrolysate including maltose, arabinose, and cellubiose, which significantly affect the acetate metabolite.

According to Liu *et al.* (2005), biohydrogen depends on the acetate (2 mol/mol glucose) metabolite, where the glucose is completely metabolized to acetate to obtain 4 mol of H_2/mol glucose. The aforementioned authors also claimed that the saccharides including arabinose, rhamnose, and others led to an increase in propionate production, which eventually affects the acetate production. Therefore, this situation has led to the lower production of biohydrogen in SPR hydrolysate as compared to sago *hampas* hydrolysate (Table 3). Therefore, based on the results of this study it can be suggested that sago *hampas* hydrolysate is a better carbon source for biohydrogen production by *C. butyricum* A1 and *C. butyricum* EB6 as compared to SPR hydrolysate. It should be noted that these two types of sago biomass hydrolysates perform better than pure glucose as a substrate for biohydrogen fermentation.

Comparison of Biohydrogen Production

The hydrogen production by *Clostridium* sp. using various types of substrates is shown in Table 4. *C. butyricum* is the most widely investigated species for biohydrogen production. This species has been tested using various types of lignocellulosic biomass and also using synthetic sugars such as glucose, sucrose, and xylose. The capability of this bacterium to consume both hexose and pentose sugars makes possible the utilization of this bacteria for biohydrogen production using lignocellulosic biomass.

Table 3. Biohydrogen Production by *Clostridium butyricum* A1 and *Clostridium butyricum* EB6 using Synthetic Glucose, sago *Hampas* Hydrolysate and Sago Pith Residue Hydrolysate

Biohydrogen production	Synthetic glucose		Sago *hampas*		Sago pith residue	
Strain	A1	EB6	A1	EB6	A1	EB6
Overall[a]	246.8	232.5	394.0	388.3	304.8	332.2
Maximum[b]	260.7	5.6	272.7	256.0	263.7	241.7
Maximum productivity (mL/L/h)	170.1	165.9	1757.4	1720.7	1643.1	1578.1
Lag phase (h)	8	10	5	3	7	7
Hydrogen percentage (%)	64	64	65	60	61	61
Biohydrogen yield (mol H_2/mol glucose)	1.9	1.79	2.65	2.50	2.23	2.11

[a]overall production of biohydrogen after 24h of fermentation time
[b]Maximum potential production estimated based on modified Gompertz equation

The theoretical biohydrogen production yield by *C. butyricum* is 4 mol H_2/mol glucose, while the highest experimented reported yield was 3.26 mol H_2/mol glucose (Keskin and Hallenbeck 2012). Other *Clostridium* spp., including *C. pasterium, C. thermolacticum, C. paraputrificum*, and *C. bifermentans* have been reported for biohydrogen production (Saratale *et al.* 2008). Mixed culture and sludge from wastewater treatment have also been employed for biohydrogen production. In that particular system, *Clostridia* were dominant for biohydrogen fermentation, showing that this bacterium is the microorganism having the greatest potential for biohydrogen production (Fang *et al.* 2002).

In addition, this study also demonstrated the capability of the locally isolated strain *C. butyricum* A1 for biohydrogen production. This strain produced a higher biohydrogen yield (1.90 mol H_2/mol glucose) than *C. butyricum* EB6 (1.79 mol H_2/mol

glucose), *C. butyricum* (Pattra *et al.* 2008), *C. pasterium* (Saint-Amans *et al.* 2001), *C. butyricum* CGS5 (Lin *et al.* 2008), and *Clostridium* sp. (Lin and Chang 2004) using synthetic sugars (glucose, sucrose, or xylose) as substrates. A higher biohydrogen yield was obtained by *C. butyricum* A1 using sago *hampas* and SPR hydrolysate compared to other fermentation, as shown in Table 4 (see Appendix). When compared to a study by Lo *et al.* (2010), the biohydrogen yield obtained was 3-fold higher, equivalent to 2.65 mol H_2/mol glucose using sago *hampas* as substrate. This value was far higher than the biohydrogen yield obtained by *C. butyricum* CGS5 using a pretreated rice straw, with a yield of 0.76 mol H_2/mol glucose (Kongjan *et al.* 2010). However, a comparable biohydrogen yield was obtained in a study done by Eriksen *et al.* (2008), where 2.6 mol H_2/mol glucose was obtained from wheat straw hydrolysate. Therefore, this study showed the potential of sago *hampas* and SPR hydrolysate to serve as cheap, abundant, and renewable substrate for biohydrogen production.

CONCLUSIONS

1. The highest cumulative biohydrogen production and biohydrogen yield were obtained when using sago *hampas* hydrolysate equivalent to 3939 mL/L-medium and 2.65 mol H_2/mol glucose, respectively.

2. Both sago *hampas* hydrolysate (2.65 mol H_2/mol glucose) and SPR hydrolysate (2.23 mol H_2/mol glucose) produced higher biohydrogen yields than synthetic glucose (1.90 mol H_2/mol glucose), which showed the potential of these two types of sago biomass to serve as fermentation substrates for biohydrogen production.

3. This study also showed a comparable biohydrogen production by the newly isolated strain *C. butyricum* A1 when compared to a well-known biohydrogen producer, *C. butyricum* EB6.

ACKNOWLEDGEMENTS

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APPENDIX: Table 4. Comparison of Biohydrogen Production by *Clostridium* sp. using Various Types of Substrates

Substrate	Microorganisms	Max hydrogen production rate (mL/L/h)	Hydrogen yield (mol H$_2$/mol substrate)	References
Glucose	*Clostridium* sp. strain No. 2	0.021[a]	2.36	Taguchi *et al.* (1996)
Glucose	*C. acetobutylicum*	na	1.97	Saint-Amans *et al.* (2001)
Sucrose	*C. pasterium*	0.214[b]	1.15	Lin and Chang (2004)
Xylose	*Clostridium* sp.	0.01[a]	1.30	Lin *et al.* (2008)
Xylose	*C. butyricum* CGS5	212	0.68 – 0.73	Lo *et al.* (2008)
Glucose	*C. butyricum* EB6	172	Na	Chong *et al.* (2009)
Glucose	*C. butyricum* EB6	158	1.79	This study
Glucose	*C. butyricum* A1	170	1.90	This study
Wheat straw hydrolysate	Extreme thermophilic mixed culture	na	0.014[c]	Kongjan *et al.* (2010)
Pretreated rice straw	*C. butyricum* CGS5	26	0.76	Lo *et al.* (2010)
Sugarcane bagasse hydrolysate	*C. butyricum*	67	1.73[d]	Pattra *et al.* (2008)
POME hydrolysate	POME sludge (seed culture)	155	2.48	Khaleb *et al.* (2012)
Sago *hampas* hydrolysate	*C. butyricum* A1	1757	2.65	This study
Sago pith residue hydrolysate	*C. butyricum* A1	1643	2.23	This study

na – not available

POME – palm oil mill effluent

[a]mol/L/h

[b]mmol-H$_2$/g-VSS/day

[c]mol H$_2$/g glucose

[d]mol H$_2$/mol total sugar