BIO-HYDROGEN PRODUCTION FROM TROPICAL BIOMASS FOR SUSTAINABLE ENERGY RESOURCES

Tsuyoshi Imai[1*], Rafiani Hasyim[2]

[1,2]Division of Environmental Science,
Yamaguchi University, JAPAN.

[*]*imai@yamaguchi-u.ac.jp*

ABSTRACT

The fermentative process of tropical biomass has found to yield hydrogen (H_2) and high rates of bacterial growth. Sago starch is commonly used in the making of many starchy food and beverages products. In general, many sago factories are located near rivers and straits where the risk of wastewater to be discharged. Therefore this study aims to investigate the fermentation of sago starch performed in different forms which can reduce the environmental impacts and recover the energy lost in the waste stream. Sago starches from Bengkalis, Indonesia were cultivated under moderate and extreme condition which includes pH, concentration and microbial community parameters for the H_2 production test. The sago fermentation in the enrichment culture shows the increment of H_2 yield by increasing the number of repeated batch cultivation. In addition, the highest hydrogen has been recovered when the pH is ranging from 6.5 to 8.0. This condition occurred when the acetic acid fermentation pathway was expected at the maximum level at 4 mol H_2/mol glucose. This study confirmed that the highest H_2 yields (maximum, 444.2 mg/g $starch_{added}$) can be observed when the starch concentration was sustained at the range of 2.5 to 15 g/L. Gelatinized dry starch was found to produce the highest H_2 yield (157.3 mL H_2/g $starch_{added}$) when compared to gelatinized wet starch, non-gelatinized dry starch and non-gelatinized wet starch. A denaturing-gradient-gel-electrophoresis (DGGE) shows no significant differences profiles from the four types of sago starch. However, all of the sago starch mixed culture show the present of Thermoanerobacterium at 49~52% GC except from the non-gelatinized wet starch mixed culture. In addition, no archeal cells have been observed in the mixture by 16S rRNA nested PCR detection method. The result of the microbial detection and the higher yield of H_2 from the gelatinized sago starch cultures indicated that gelatinized culture contains microorganism that able to simultaneously degrade starch and produce H_2 efficiently.

Keywords: tropical biomass; environmental impact; bio-hydrogen

1. INTRODUCTION

Production of bio-hydrogen by fermentative processes is desirable because such methods generate high yields of hydrogen and high rates of bacterial growth with relatively low energy inputs, compared to the photobiological methods [1-2]. Not only is hydrogen a clean energy carrier, some bio-hydrogen production processes are environmentally sustainable. In particular, fermentation processes that utilize free carbon available in large-volume discharges of agro-industrial wastewater containing carbohydrates or lignocellulosic materials can recover available energy as well as purify the effluent [3-8]. In addition, high-temperature waste streams such as those from food processing plants are especially conducive to hydrogen-producing reactions, because of the thermodynamics of the reaction processes [9] and the resistance of these waste streams to contamination by pathogens [10-13]. O-Thong et al. [1] have reported that thermophilic bacteria can utilize a variety of carbon sources and generate high yields of hydrogen as well as tolerate acidic fermentation conditions. Thermophilic anaerobic fermentation processes hold tremendous promise for the forthcoming generations as well as for the commercial production of hydrogen fuel and concomitant purification of waste streams.

The capacity of fermentative microorganisms to produce hydrogen from carbohydrates is well known and has been studied and applied in a wide range of applications [14]. Researchers have utilized a variety of carbohydrates for fermentation, from simple sugars, including glucose [15] and sucrose [16], to more complex carbohydrates such as starch and carbohydrate-rich agricultural products such as rice [17-18]. Researchers have also investigated the production of hydrogen from carbohydrate-rich wastewaters to benefit the economy and to support the Clean Development Mechanism (CDM) [19]. Hydrogen has been produced by fermentation of the wastewater from a sugar factory [20], a sweet potato-starch manufacturing plant [21], and a brewery [22]. In this study, data and observations will be presented on the production of hydrogen from sago starch in wastewater.

Starch from the sago palm (*Metroxylon sagu* Rottb.) has long been a staple food for populations in the Moluccas, West Papua (Indonesia) and Papua New Guinea, and is widely used as a dietary supplement in Southeast Asia. In food industries, sago starch is used as an ingredient while making noodles, vermicelli, biscuits, monosodium glutamate, glucose, caramel, fructose, syrups, and many other food products [23]. Sago palm is considered a potential commercial crop because of its numerous benefits compared to the other starch crops, including high starch yields (15–25 ton/ha of dry starch), relatively sustainable production, vigor, environmental friendliness [24], and low cost of production [25]. On Bengkalis Island and the surrounding areas of Riau Province in Indonesia, starch is already being produced by the local sago industries, and palm is being cultivated for large-scale commercial production [26]. Moreover, Bustaman [27] had documented the potential for sago-based bioethanol production in Moluccas, and several other studies have studied the production of bioethanol from this starch [28-30]. However, few studies have used sago starch or the wastewater from the sago starch-manufacturing units for biogas production [25] and no report for hydrogen production.

Sago starch factories are usually located near rivers or straits, and many of them discharge wastewaters containing starch and pith residue directly into the waterways without proper treatment. Production of wastewater in an average facility can reach 10^6 l/day, with a biological oxygen demand (BOD) and chemical oxygen demand (COD) of 3.4 g/l and 11.4 g/l, respectively [31]. BOD and COD are directly attributed to starch and

pith residues. Pith residues or "*hampas*," containing about 60–70% dry weight starch, are a by-product of the rinsing procedures; approximately 1 ton/day of *hampas* is generated by an average factory [25,32].

To reduce environmental impacts and recover the energy lost in the waste stream, we investigated the feasibility of using sago starch in wastewater for fermentative hydrogen production by using a thermophilic mixed culture from a hot spring in Thailand. A mixed culture from a hot spring was selected because hot springs are recognized as a potential source of thermophilic microorganisms with fermentative abilities, such as hydrogen-producing bacteria [33-34]; also, mixed cultures have benefits in industrial applications because of their high microbial diversity, and because media sterilization is not required [6,35].

In this study, the researcher examined the effects of repeated batch cultivations, the use of different initial conditions of pH, sago starch concentration, and starch type on biohydrogen production in 4 serial batch experiments conducted at 60°C. In addition, the researcher investigated the microbial community of the thermophilic mixed culture in the sago starch media by using 16S-rRNA-based molecular techniques, including polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing.

2. MATERIALS AND METHODS

2.1 The Mixed Cultures from a Hot Spring and Anaerobic Digested Sewage Sludge

The mixed culture from Khong Pay Pao hot spring (PGK) used in this study was obtained from previous research conducted by Hniman et al. [36]. The culture was enriched from sediment-rich water sample collected from a geothermal hot spring located in Khong Pay Pao, Phang Nga, Southern Thailand. Temperature and pH of sample were 60 °C and 6.5, respectively. The enrichment of culture in basic anaerobic (BA) medium supplemented with 10 g/l each of glucose, xylose and xylose-glucose mixed substrate at the ratio of 1:1 under strictly anaerobic condition at 60 °C was performed by repeated batch cultivations following the procedure of O-Thong et al. [37] as described in detail by Hniman et al. [36]. This culture gave maximum hydrogen yield of 199.8 ml-H_2/g-$sugar_{consumed}$ using glucose-xylose mixed substrates. Subsequently, this culture was used in this study for producing hydrogen from sago starch in wastewater.

Anaerobic digested sludge was collected from Ube City Municipal Wastewater Treatment Plant, Western Purification Center, Ube City, Yamaguchi Prefecture, Japan. The initial pH, SS and VSS of the sludge were 7.02, 11.7, and 8.5 g/l, respectively.

2.2 Preparation of Sago Medium

Bushnell-Haas medium (BHM) consisting of (g/l): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 1; KH_2PO_4, 1; NH_4NO_3, 1.0; $FeCl_3.6H_2O$, 0.05; $CaCl_2$, 0.02 supplemented with yeast extract (1.0 g/l), peptone (1.0 g/l) and sago starches were prepared for further batch hydrogen production. Sago starches, wet and dry types, from Bengkalis, Indonesia were supplied by local producer which were processed for commercial uses. Gelatinization of those starches was performed by autoclaving at 121 °C for 15 minutes to compare with the raw starches.

2.3 Batch Hydrogen Production under Moderate- and Extreme-Thermophilic Conditions

2.3.1 Hydrogen Production by PGK Culture

In batch fermentation, the effects of repeated batch cultivations, the effects of different initial pHs, initial sago starch concentrations and starch types on biohydrogen production from sago starch in wastewater by enriched PGK culture in 4 serial experiments were investigated. For all experiments, 50 ml modified BHM supplemented with sago starch as substrate was distributed anaerobically in 125 ml serum bottles capped with rubber stopper and closed with aluminum caps. The 5 ml of enriched PGK culture was transferred into the medium and after the headspace was replaced with nitrogen gas, the mixture was cultivated at 60 °C for 3 days. The initial VSS concentration measured in each serum bottle was around 90 mg/l. The pH was set to 6.5 in all experiments, except for the second series. The initial concentration of sago starch was adjusted to 10 g/l except for the third series and gelatinized dry starch was used for all experiments except for the fourth series. All experiments were performed triplicate for verification purposes. The detail procedures for each serial experiment are explained below.

First, to determine the effects of repeated batch cultivations, 5 ml of enriched PGK culture was cultivated in 50 ml medium at 60 °C for 3 days. Then, 5 ml of inoculum from the first batch was taken for cultivating into the second batch. This procedure was repeated until 6 batch cultivations under identical conditions. Second, to determine the dependence of initial pH, the pH was set to various levels (in the range of 5.0–8.0, with increments of 0.5) in 7 serum bottles using 1 N HCl and 1 N NaOH. Third, to verify the dependence of initial sago starch concentration, the experiment was started with the initial concentrations ranging from 10 to 60 g/l at increments of 10 g/l in 6 serum bottles. Afterward, lower range concentration of sago starch (from 2.5 to 15 g/l with increments of 2.5) was set in another 6 serum bottles to confirm the result. In the fourth series, in order to observe the effects of sago starch type, we used 10 g/l of gelatinized and non-gelatinized of dry and wet starches in 4 serum bottles.

2.3.2 Hydrogen Production by Heat Treated Anaerobic Digested Sewage Sludge Culture

The experiments were conducted in 3 repeated batch cultivations. For the first run, 60 ml of anaerobic digested sludge (VSS=8.5 g/l) was prepared in 125 ml serum bottle and the headspace was purged with nitrogen gas. Heat shock pretreatment of sludge (105 °C, 20 min) was performed by the same method as described in the previous chapter. The media containing gelatinized dry sago starch as substrate was injected until the final concentration of 2 g/l was achieved. The initial pH was set to around 5.5. Then, all batch

tests were incubated in incubator at 60 ºC (moderate thermophilic condition) and 70 ºC (extreme-thermophilic condition) for approximately 4 days. The second run was started by the injection of new media (final starch concentration of 2 g/l) into previous batch and cultivated for approximately 4 days. For the third run, the previous culture was centrifuged and all of liquid phase was removed to avoid the inhibition of H_2 production caused by the accumulation of organic acids. The sludge remained was mixed with new media solution (working volume = 60 ml, starch concentration = 2 g/l) and then cultivated with the same condition as the previous run. The sludge and liquid samples (5 ml) were taken at the initial and the end of each run for microbial analysis and metabolites composition. For comparison, similar experiment was also conducted with glucose as substrate.

2.4 Analytical Methods

Biogas volume was measured daily with a glass syringe and the composition was analyzed by gas chromatography. Liquid samples were also taken from the culture before and at the end of each experiment for analyzing the composition of soluble metabolites including pH, volatile fatty acids (VFA), total organic carbon (TOC), reducing sugar, and total carbohydrates. The measurement condition for biogas, VFA, and TOC analysis were similar with our previous report [38]. Concentration of reducing sugar and total carbohydrates were determined by DNS [39] and anthrone-sulfuric acid methods [40] using a spectrophotometer U-2001 (Hitachi, Japan) at 540 nm and 620 nm, respectively. Volatile suspended solid (VSS) was quantified according to the standard methods for the examination of water and wastewater [41].

2.5 Microbial Community Analysis

The procedure of microbial analysis was depicted in Figure . PCR-DGGE was used to study microbial community structure in two-stage process. Liquid samples were collected from serum bottles under steady state conditions. The microorganisms cells in 2 ml of sample were harvested in a tube by centrifuged at 5,000g for 5 min. The pelletized cells were washed three times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) before being resuspended in 1 ml of TE buffer. Genomic DNA was extracted and purified using QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany). Genomic DNA was used as a template for PCR reactions with a primer pair specific for *Eubacteria* (universal primer 1492r and primer 27f) as well as for *Archeae* (Arch21f and Arch958r) [42]. Each 50 µl (total volume) reaction mixture containing 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 5 mM $MgCl_2$, each deoxynucleotide triphosphate at a concentration of 200 mM, 1 µl of Taq polymerase (2U/ml; Sigma-Aldrich, St. Louis, MO), 10 pmol of each primer, and 1 µl of DNA extract solution. Sterile water was used as no template control, 1 µl genomic DNA from *Caldicellulosiruptor saccharolyticus* and *Sulfolobus islandicus* as positive and negative control, respectively. The thermal cycling program used for first amplification was as follows: predenaturation at 95ºC for 5 min; 30 cycles of denaturation at 95 ºC for 30 s, annealing at 52ºC for 40 s and elongation at 72ºC for 90 s. The reactions were subsequently post elongation at 72ºC for 30 min and cooled to 4ºC. Amplification was checked by agarose gel electrophoresis of PCR products using 1% agarose in 1xTAE buffer. First PCR products were used as a template for nested PCR reactions with a primer pair specific for *Eubacteria* (Primer 518r and 357f with 40 bp GC clamp at the 5' end; [43]) as well as for *Archeae* (PARCH519R and PARCH340F with 40 bp GC clamp at the 5' end; [44]) were used to amplify the 200 bp fragment of the V_3 region. The amplicons were used as DNA template to incorporate a GC clamp in the DNA fragment

prior to DGGE [45]. The second PCR program corresponded to 20 cycles of three steps: 94ºC for 1 min, 65ºC for 0.75 min, and 72ºC for 1 min, 10 cycles of three steps: 94ºC for 1 min, 55ºC for 0.75 min, and 72ºC for 1 min followed by a final step at 72ºC for 10 min. Amplification was checked by agarose gel electrophoresis of PCR products before DGGE analysis.

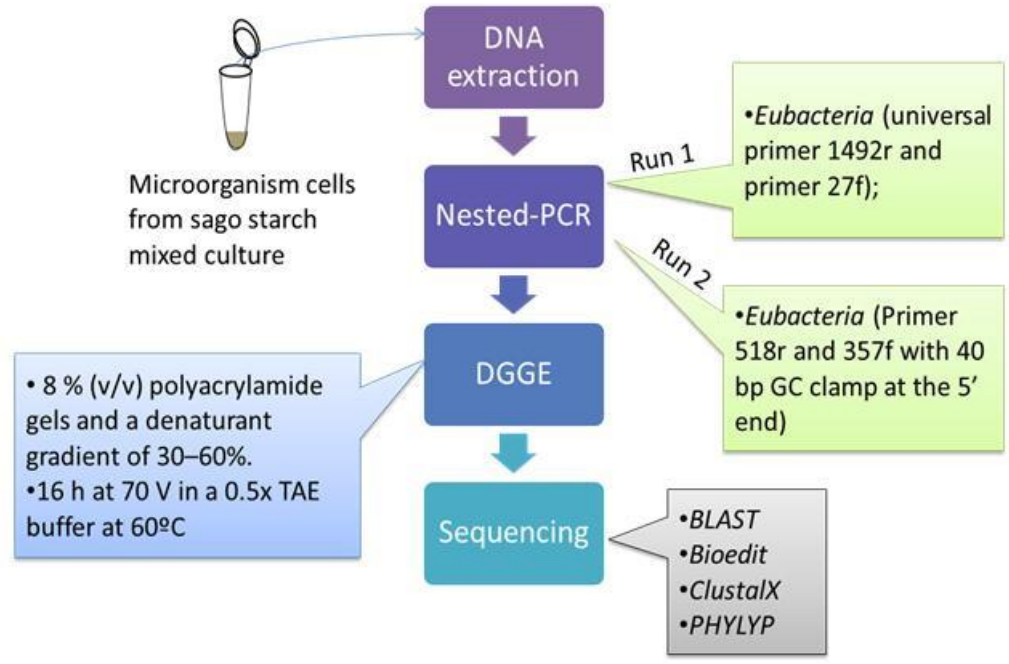

Figure 1: The procedure of microbial analysis using PCR-DGGE

DGGE analysis of the amplicons obtained from second PCR was performed as previously described by Zoetendal et al. [46] using the Dcode Universal Mutation Detection system (Bio-Rad, Hercules, CA) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30–60%. A 100% denaturing solution was defined as 7 M urea and 40% formamide. Electrophoresis was performed for 16 h at 70 V in a 0.5x TAE buffer at 60ºC. The DGGE Marker II set (Nippon Gene, Tokyo, Japan) was co-electrophoresed with the samples. DGGE gels were stained with SYBR Green for 15 min and analyzed on GelDoc XR 1708170 system (Bio-Rad Laboratories, Hertfordshire, UK). DGGE profiles were compared using the Quantity One software package (version 4.6.0; Bio-Rad Laboratories). Most of the bands were excised from the gel and re-amplified with primer 357f without a GC clamp and the reverse primer 518r. After re-amplification, PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and sequenced using primer 518r and were directly sequenced (Macrogen; http://www.macrogen.com). Closest matches for partial 16S rRNA gene sequences were identified by database searches in Gene Bank using BLAST [47].

2.6 Fed Batch Hydrogen Production in 2L Jar Fermentor

A CSTR-type (completely stirred tank reactor-type) reactor (Jar Fermentor MBF; Eyela Tokyo Rikakikai Co. Ltd., Tokyo, Japan) with a working volume of 1.5 l was used as the hydrogen fermentation reactor for the upscale experiment (Fig). A 1.2 l of heat treated anaerobic digested sewage sludge, provided by Ube City Municipal Wastewater Treatment Plant, Japan was added to the reactor and 0.3 l. of media containing gelatinized dry sago starch was injected with the final concentration in the reactor was 5 g/l. The initial pH was adjusted at 5.5 by addition of 2N HCl solution, and the temperature and agitation speed were maintained at 70°C, and 200 rpm, respectively. The generated biogas was channeled directly into a gas collection bag and the volume of collected gas was measured by water displacement method. For analyzing the gas composition, 0.5 ml of produced biogas was taken at designated time intervals and measured by gas chromatography. To compare the performance of hydrogen productivity, similar experiment was conducted by using PGK culture.

Figure 2: Bioreactor used in this experiment

3. RESULTS AND DISCUSSION

3.1 Hydrogen Production from Sago Starch Wastewater by Mixed Culture from a Hot Spring

3.1.1 Biogas Composition and the Effects of Repeated Batch Cultivation

The composition of biogas measured in all series of our experiments was approximately 40–55% hydrogen and 45–60% carbon dioxide (excluding the residual nitrogen from the initial sparging). No methane was detected in any treatment, verifying the merit of an enriched PGK culture for biohydrogen production, as previously reported [36]. The utilization of this enriched culture for the fermentation of more complex carbohydrates, namely, sago starches, yielded promising results. We found that hydrogen yields were increased by increasing the number of repeated batch cultivations until a nearly stable level of hydrogen production was achieved, as shown in Fig. The advantage of repeated batch cultivations is that it allows the microorganisms in a culture to adapt to their environment, resulting in increased hydrogen yields as compared to single batch cultivations described in previous reports [1,48].

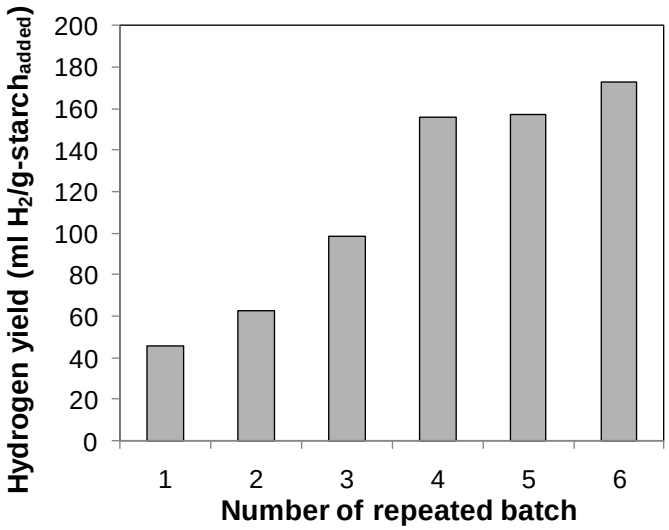

Figure 3: Hydrogen production at different batch cultivations with 10 g/l initial gelatinized dry sago starch concentration using enriched PGK culture at 60 °C

3.2 Dependence of Biohydrogen Production on Initial Ph

Initial pH has been recognized as an important determinant of biohydrogen productivity. Table 1 summarizes the effect of initial pH levels on final pH, H_2 yields (ml/g-starch$_{added}$), cell yields (g-VSS/g-starch$_{added}$), VFA yields (g/l), reducing sugar content (g/l), and substrate removal (%) for 10 g/l gelatinized dry sago starch fermented for 3 days at 60°C. The data indicate that hydrogen production is high for initial pH values of 6.5–8.0, and that maximum H_2 yields are obtained at pH 6.5. The final pH under conditions of high hydrogen yield decreased to approximately 4.5 because of the accumulation of VFAs. Acetic acid was the sole VFA product at all pH values, indicating that the enriched PGK culture produced H_2 via the acetic acid fermentation pathway, which is known to produce more hydrogen than other pathways. As summarized by Hasyim et al. [38], fermentation reactions producing hydrogen from glucose can occur by several pathways: acetic acid fermentation, butyric acid fermentation, ethanol fermentation, and a mixture of acetic acid

and butyric acid fermentation. Only acetic acid fermentation can produce the maximum levels of hydrogen generation (4 mol H_2/mol glucose), whereas the other fermentation mechanisms can produce only 2 mol H_2/mol glucose.

The fermentation culture with an initial pH of 6.5 also attained the highest cell yield of all trials, confirming that this pH is preferable for the growth of starch-hydrolyzing and hydrogen-producing microorganisms in this culture. Interestingly, the maximum substrate utilization was attained at pH 7–8, not at pH 6.5, implying that at least one other important metabolite is also produced during fermentation. Ethanol fermentation is a possible route for producing H_2 within this pH range, because of the lower H_2 yields. Other metabolites that might be present in this pH range are alcohols and lactic acid, as shown by Hniman et al. [36] in their study using PGK cultures. Lee et al. [49] found that limiting the pH to 5.5–7.0 inhibits the production of ethanol in the H_2-producing fermentation of cassava starch. Therefore, adjustment of the initial pH to values of approximately 6.5 is considered important for the production of hydrogen from sago starch using the hot spring culture from Thailand.

Table 1: Hydrogen production from sago starch using PGK culture at stationery phase of different initial pH values

Initial pH	Final pH	H_2 yield (ml/g-starch $_{added}$)	Cell yield (g-VSS/g-starch $_{added}$)	VFA (g/l)			Reducing Sugar (g/l)	Substrate removal (%)
				HAc	HPr	HBu		
5	4.9	3.71	0.08	0	0	0	1.13	10.35
5.5	5.3	2.96	0.08	0	0	0	5.63	46.51
6	5.9	1.59	0.06	0.25	0	0	2.57	27.54
6.5	4.4	62.83	0.14	1.11	0	0	0.68	79.51
7	4.5	45.15	0.06	1.08	0	0	1.44	91.20
7.5	4.4	30.50	0.09	0.77	0	0	0.78	91.89
8	4.6	32.49	0.07	0.86	0	0	0.88	90.24

At an initial pH of 5.0–6.0, hydrogen yields in our trials were very low compared to the yields at higher pH levels. Low pH fermentation was accompanied by lower substrate utilization and undetected VFAs (Table), indicating that H_2 production is inhibited at low pH. Similarly, Zhang et al. [17] reported low hydrogen yields at pH 5, and no hydrogen production at pH 4. On the other hand, Lin et al. [50] found that initial pH values of 5.0–5.5 were more favorable than higher pH levels for hydrogen production from the starch in a paper mill-wastewater treatment sludge digested by a natural mixed culture, suggesting that the microflora in this environment are preferentially adapted to low pH conditions. Thus, inconsistent results regarding the effects of initial pH on hydrogen production from starch are probably because of the different characteristics of the microorganisms in the cultures and due to the variations in the substrate environment. Hence, it is necessary to study the effects of initial pH on hydrogen production from diverse environments and cultures before establishing large-scale hydrogen production systems.

At low pH values, especially at a pH of approximately 5.5, relatively high levels of reducing sugars were observed (5.63 g/l) in our trials (from initial reducing sugar concentrations of approximately 0.3 g/l). This indicates that the hydrogen-producing microbe in the culture is not active in these conditions (indicated by the low hydrogen yield), but that the starch-hydrolyzing microorganism in the culture still demonstrates high amylase activity. Konsula and Liakopoulou-Kyriakides [51] examined the effects of pH on the activities of the enzymes involved in starch hydrolysis, and found that the enzyme involved in amylase production displayed considerable activity in the pH range of 5–7.5, and optimal activity at pH 6.5. This might explain the observation made that hydrogen production is highest at pH 6.5, but that reducing sugars are still produced at other pH values.

3.2.1 Dependence of Biohydrogen Production on Initial Sago Starch Concentrations

In addition to pH and substrate composition, substrate concentrations are recognized as one of the critical factors in the production of hydrogen. Biological metabolic processes, including specific metabolic pathways and the reaction kinetics of hydrogen production, are considered to be affected by substrate concentrations [49,52]. The measurements of hydrogen yield in response to the amount of available substrate (starch) provide valuable information about the potential of the substrate to generate hydrogen. Therefore, many research studies report the efficiency of hydrogen production as a function of the hydrogen produced per amount of substrate added [6,17].

In this study, the researchers examined optimal hydrogen production efficiency as a function of the initial sago starch concentration. In the first set of trials, the researchers used initial starch concentrations ranging from 10–60 g/l, in increments of 10 g/l. Figure 1 illustrates H_2 yield (ml/g-starch$_{added}$), substrate removal (%), reducing sugar content (g/l), and cell yield (g-VSS/g-starch$_{added}$) at different initial sago starch concentrations, digested for 3 days at 60°C and an initial pH of 6.5. Figure 1B shows that hydrogen yield and substrate removal tend to decrease at sago starch concentrations greater than 10 g/l.

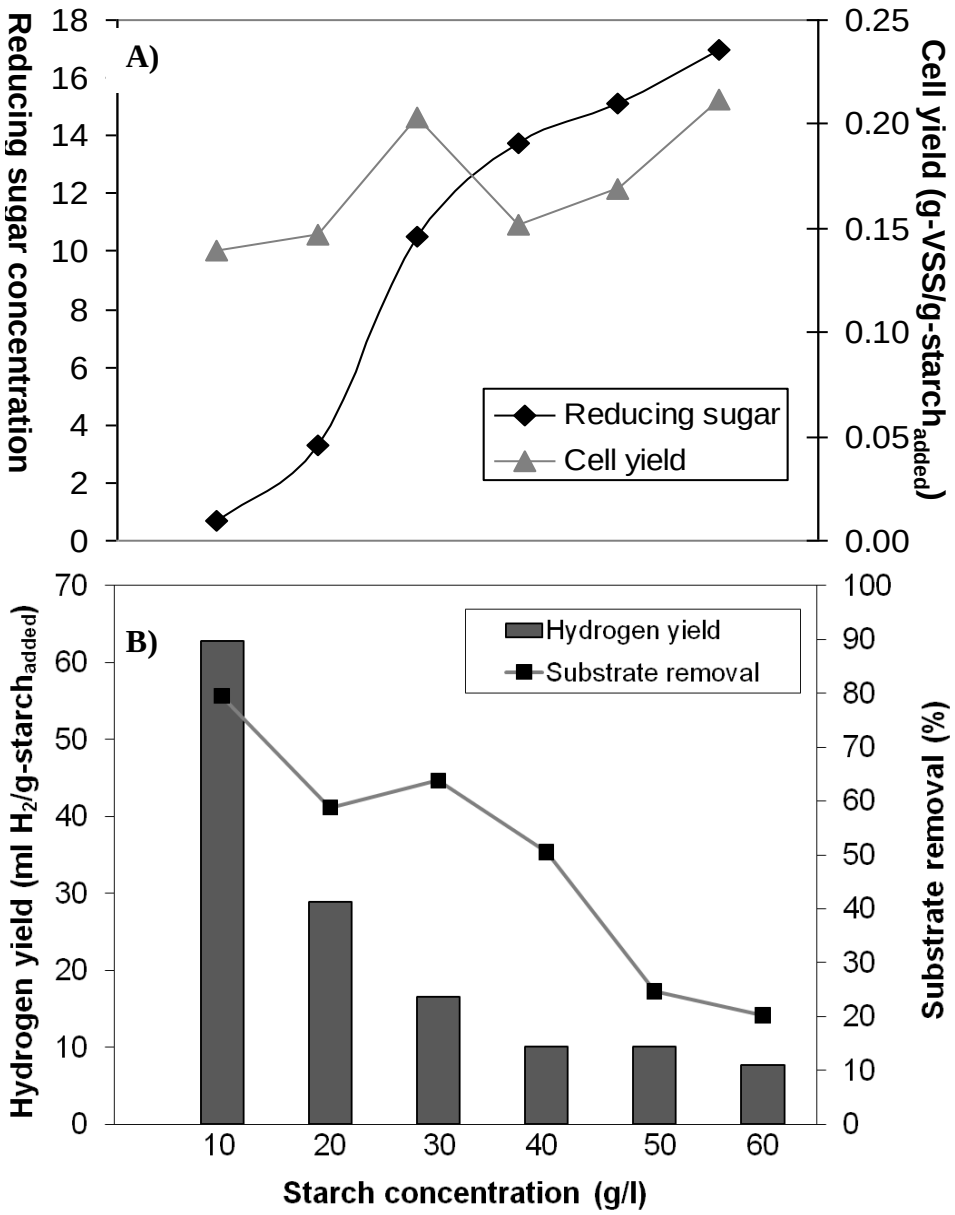

Figure 1: Hydrogen production from sago starch using PGK culture at initial starch concentration of 10-60 g/l after 3 days cultivation (A) Reducing sugar concentration and cell yield (B) H_2 yield and substrate removal

Further investigations using lower initial starch concentrations (2.5–15 g/l) showed that higher hydrogen yields (maximum, 442.2 ml/g-starch$_{added}$) were achieved at initial sago starch concentrations of 2.5 g/l (Table 1). This hydrogen yield is nearly 80% of the theoretically derived maximum value of 553 ml of hydrogen per gram of starch [17]. To date, such efficiencies have never been reported in other studies on hydrogen production from starch. The high yield of hydrogen at low initial starch concentrations was concomitant with the consumption of nearly 100% of the starch, as shown in Table 1. Acetic acid was the sole VFA product under these conditions (data not shown), confirming that starch is digested via the acetate fermentation pathway.

Table 1: Starch balance and hydrogen yield from sago starch using PGK culture at static state of different initial starch concentrations (2.5-15 g/l)

Initial conc. (g/l)	Starch balance				H_2 yield (ml/g-starch$_{added}$)	Final pH
	input (mg)	remain (mg)	consumed (mg)	removal (%)		
2.5	125	0	125.00	100	442.2	4.69
5.0	250	67.53	182.47	72.99	276.4	4.02
7.5	375	177.53	197.47	52.66	236.2	3.88
10.0	500	350.19	149.81	29.96	156.1	3.93
12.5	625	354.37	270.63	43.30	111.6	4.39
15.0	750	269.43	480.57	64.08	91.5	4.36

Final pH levels in all conditions were approximately 4, due to the release of organic acids during the fermentation process. Lower hydrogen yield at higher initial starch concentrations have been observed by several researchers, and this is possibly caused by factors such as incomplete hydrolysis of starch, increase in the concentration of byproducts (such as VFAs and alcohols), accumulation of undissociated organic acids, or inhibition of the microorganisms involved in starch digestion or hydrogen production [6,8,17,52]. These factors might explain why lower initial concentrations of sago starch are favorable for hydrogen production, as observed in our study.

Figure 1A shows that the reducing sugar concentration after 3 days of cultivation tends to increase with increasing sago starch concentrations, indicating that the starch-degrading capacity of this culture is quite high. Once again, this result may be possibly due to the high amylase activity of starch-degrading microorganisms in the culture, as well as the effects of temperature (60°C). It can also be considered that a higher temperature of cultivation would facilitate the degradation of starch into simple sugars, as compared with lower temperatures, because starches start to swell at 55°C or higher depending on the type of starch, pH, water content, and other factors [53]. Hence, cultivation under thermophilic conditions is recommended for hydrogen production from starch, including sago starch, because the starch gelatinization temperature is around 70°C [24].

Figure 1A also shows that low reducing sugar contents accompany higher hydrogen yields that are produced from an initial starch concentration of 10 g/l (Figure 1B), as compared to other initial starch concentrations, indicating that sugars in the medium are consumed by the hydrogen producers in the culture. The high hydrogen yields are also accompanied by the highest percentage of substrate removal. Cell yield at an initial starch concentration of 20 g/l were similar to the yield at 10 g/l, but with lower hydrogen yield, lower substrate utilization, and higher sugar concentration. Cell yield was substantially increased at an initial starch concentration of 30 g/l, paralleled by an increasing concentration of reducing sugars. In summary, these observations suggested that higher initial starch concentrations do not promote the growth of hydrogen-producing microbes in this culture; nevertheless, the results reveal the potency of starch degraders to grow.

3.2.2 Comparison of Biohydrogen Production from Different Sago Starch Types

Two types of starch are commercially produced in sago starch manufacturing factories, namely, wet and dry. The main difference between wet and dry starch is the moisture content. In addition, the structure of starch may change because of spontaneous fermentation during long-term storage. Wet starch cannot be stored without proper storage methods because microbial activity may corrode the grain, thus diminishing the quality of the starch [24,54]. Regional variation is observed in the traditional methods of storing wet starch.

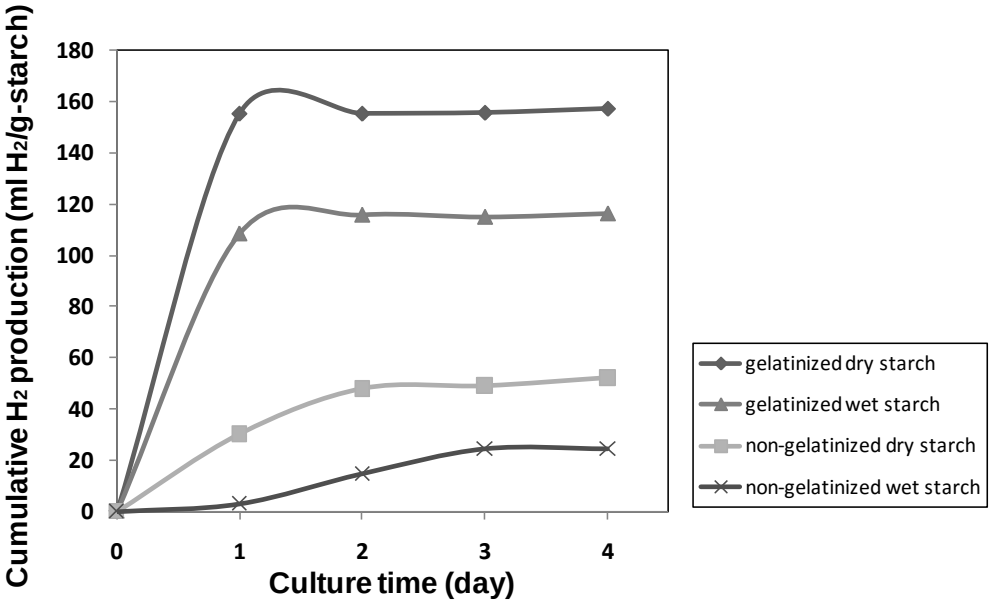

Figure 2: Hydrogen production from sago starch using PGK culture at different starch type with 10 g/l initial starch concentration for 4 days cultivation

In this study, each type of starch was gelatinized at 121°C for 15 min to examine the effects of starch gelatinization on bio hydrogen production; results were compared with the data on raw starch. The cumulative hydrogen yields (ml H_2/g-starch$_{added}$) from the different types of sago starch for 4 days of cultivation with 10 g/l initial starch concentrations are depicted in Figure 2. H_2 production ceased after 2 days of cultivation, indicating the short hydraulic retention time of the culture, probably caused by substrate- or product-induced inhibition. The highest hydrogen yields were achieved from gelatinized dry starch (157.3 ml H_2/g-starch$_{added}$); hydrogen productivity decreased when using gelatinized wet starch, non-gelatinized dry starch, and non-gelatinized wet starch (H_2 yields of 107.4, 52.2, and 8.5 ml H_2/g-starch$_{added}$, respectively). From these data, it appears that the highest hydrogen yields are obtained using enriched cultures and gelatinized dry starch.

Hydrogen yields obtained from both dry and wet gelatinized starch were higher than the yields obtained from non-gelatinized starch, clearly indicating that gelatinization favors hydrogen production. Starch gelatinization can cause irreversible changes in grain properties such as granular swelling, native crystallite melting, loss of birefringence, and starch solubilization [55-56]. Thus, gelatinization might create favorable conditions for microbial starch consumption in PGK cultures. Lower hydrogen yield from wet starch was possibly due to the properties of the starch. Further investigation is required to

completely understand the chemical and physical properties of starch and to study the effects of gelatinization on hydrogen production**.**

3.2.3 Comparison of Hydrogen Yield from Starch, As Determined In Previous Studies

The utilization of starch as a substrate for hydrogen production has been described in many research studies. Starch contains approximately 20–25% linear and helical amylose and 75–80% branched amylopectin [57]. Sago starch generally contains 27% amylose and 73% amylopectin [58], with minor amounts of protein [55]. The structure and composition of the molecules constructed by these 2 major high-molecular-weight carbohydrate components are characteristic of each particular plant species [59-60]. Therefore, different sources of starch will result in different hydrogen yields; yields also depend on factors such as the structure and composition of the microbial community, composition of the medium, and temperature.

Table 2 compares the hydrogen yields obtained by anaerobic fermentation of soluble starch, cornstarch, and cassava starch from previous studies [8,17,61-62] and sago starch (this study). The data show that the highest hydrogen yields are obtained using sago starch with initial starch concentrations of 2.5 g/l. Hydrogen yields using initial concentrations of 5 g/l sago starch (276 ml H_2/g-$starch_{added}$) can be compared to the yields obtained using other starch sources. These results demonstrate the potency of the sago starch in wastewater for producing hydrogen by using an enriched PGK culture at moderate thermophilic conditions (60°C) and an initial pH of 6.5.

Table 2: A comparison of hydrogen yields obtained by anaerobic fermentation of soluble starch, cornstarch, and cassava starch from previous studies and sago starch (this study)

Starch source	Initial Conc. (g/l)	Temp. (°C)	pH	Organism	Reactor	H_2 yield (ml/g starch)	Ref.
Starch in wastewater	4.6	55	6	H_2 -producing sludge	Batch	92	[17]
Corn starch	2	35	8	Mixed bacteria	Batch	194	[61]
Cassava starch	10	35	7	Anaerobic activated sludge	Batch	240	[62]
Starch wastewater	5	37	6.5	Municipal WWTP sludge	Batch	186	[8]
Sago starch in wastewater	2.5	60	6.5	Enriched mixed culture (PGK)	Batch	442	This study

3.3 Microbial Communities of Sago Starch Thermophilic Mixed Cultures *PGK Culture*

Bacterial cultures were collected from 4 serum bottles under steady state conditions, each containing a different type of sago starch, i.e., non-gelatinized dry starch, gelatinized dry starch, non-gelatinized wet starch, and gelatinized wet starch. Figure 8 shows the DGGE profiles of 16S rRNA gene fragments for enriched PGK cultures from each bottle. A rapid comparison of the DGGE patterns of PCR products from each culture did not reveal any significant differences between the bacterial community structures in these samples, except for the communities from the non-gelatinized wet sago starch. Thermophilic anaerobic bacteria of the genus *Thermoanaerobacterium* were the dominant member in 3

of the 4 cultures (all except the non-gelatinized wet starch culture), with the strongest band intensity affiliated with the uncultured *Thermoanaerobacterium* sp.

Further observations of the DGGE profiles from the gelatinized and non-gelatinized sago starch cultures indicate that cultures from gelatinized starch (both dry and wet) possess similar microbial community structures, which are affiliated with Gram-positive bacteria, uncultured *Thermoanaerobacterium* sp., *T. saccharolyticum* and *T. thermosulfurigenes*. *Thermoanaerobacterium* sp., an anaerobic spore-forming thermophilic microbe, known for its capacity to utilize various types of substrates for hydrogen production [1]. The saccharolytic fermentative bacterium *T. saccharolyticum* participates in interspecific hydrogen transfer in its natural environment, thereby producing hydrogen from carbohydrates. The species has been engineered to produce high yields of ethanol from a wide array of biomass-derived sugars [63]. *T. thermosulfurigenes*, also known as *Clostridium thermosulfurigenes*, produces an extracellular thermoactive thermostable β-amylase and a cell-bound glucoamylase as the major amylolytic enzymes responsible for starch degradation. It also produces ethanol, acetate, lactate, and hydrogen as the main end products [64].

In non-gelatinized dry starch cultures, *Geobacillus* sp. was observed along with *Thermoanaerobacterium* sp. *Geobacillus* sp. is widely distributed, and has been successfully isolated from geothermal environments. *Geobacillus* sp. has aroused interest in the industrial sector, on account of its potential applications in the biotechnological processes, for example, as sources of thermostable enzymes such as proteases, amylases, lipases, and pullanases [65]. In the non-gelatinized wet starch culture, bands related to the *Bacillus* sp., including *B. lentus* and *Anoxybacillus* sp., were detected. These thermophilic bacilli are generally isolated from geothermal hot springs, and mainly produce amylolytic enzymes capable of utilizing carbohydrates, including starch; some can also produce hydrogen [1,66-67].

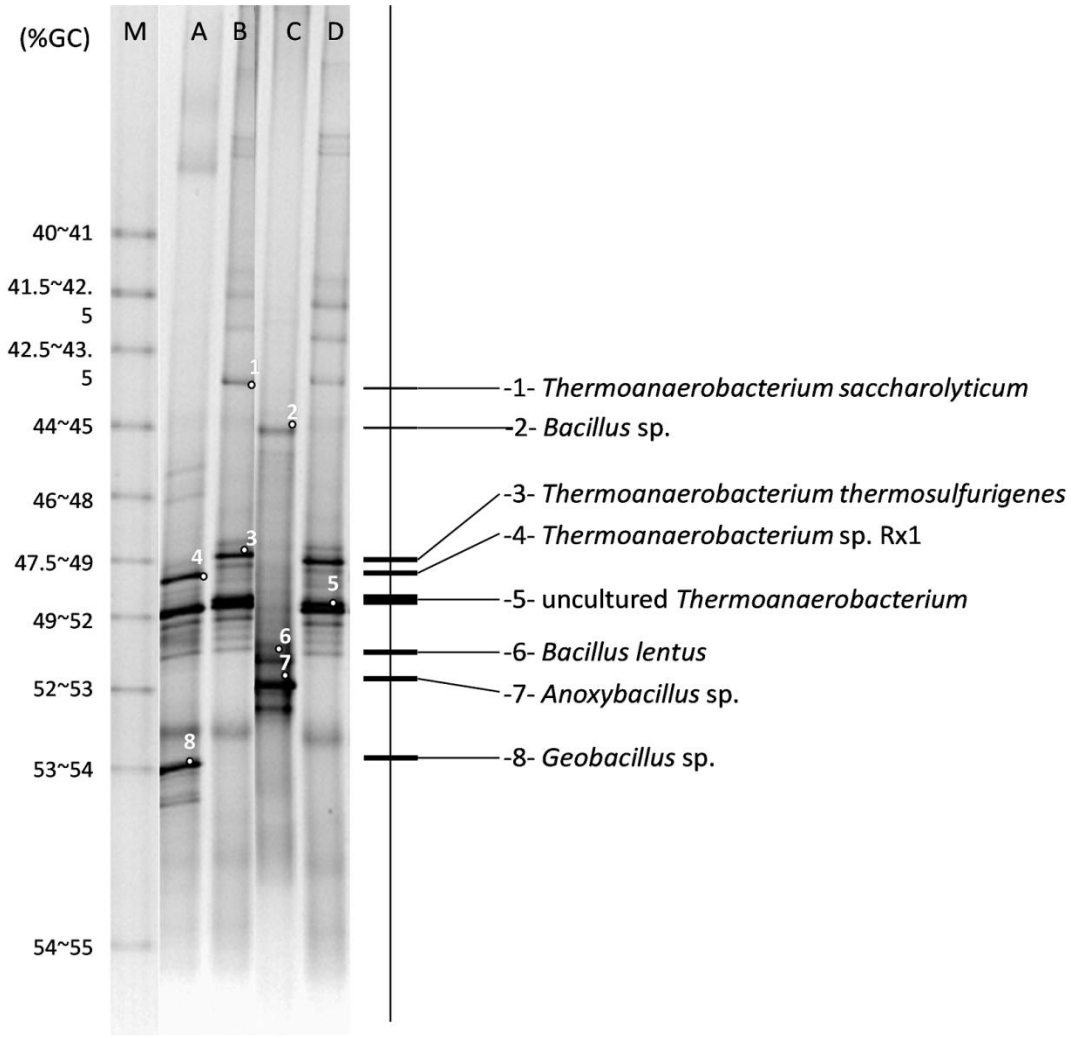

Figure 8: DGGE profile of 16S rRNA gene fragments. The fragments were PCR-amplified from total DNA extracted of enriched PGK culture with sago starch used for hydrogen production (M, DGGE marker; A, bacteria community in non-gelatinized dry sago starch; B, gelatinized dry sago starch; C, non-gelatinized wet sago starch; and D, gelatinized wet sago starch)

Since lower hydrogen yields were observed in the non-gelatinized wet starch cultures (Figure 2), we suggest that the bacteria in these cultures are associated with the starch-hydrolyzing processes, rather than with hydrogen production, as indicated by the high percentage of starch utilization (data not shown). Another possible contribution of the *Bacillus* sp. observed in this system is oxygen consumption, thereby generating the anaerobic conditions required by the hydrogen-producing microbes. The presence of some members of the aerobic *Bacillus* sp. in a hydrogen production system, in conjunction with some members of the anaerobic *Clostridium* sp., was observed in previous studies also, and is considered to positively contribute to the conditions essential for hydrogen production (an "added-value") by mixed culture methods [68-70].

The result of this microbial community analysis in combination with the higher hydrogen yields obtained from the gelatinized sago starch cultures (as depicted in Figure 2) indicate that gelatinized cultures possess microorganisms capable of simultaneously degrading starch and efficiently producing hydrogen. In conclusion, we state that the enriched mixed

culture from the hot spring in Southern Thailand (i.e., the PGK culture) shows promising potential for application in bio hydrogen production from starch; the bacterial activity simultaneously degrades starch and produces hydrogen. Nested PCR of the DNA extracts of the enriched culture samples did not detect any amplification products of the archaeal 16S rRNA genes, thus indicating the absence of archaea in the enriched cultures.

4. CONCLUSIONS

The enriched PGK culture used in this study can produce a methane-free biogas with a hydrogen content of up to 55%. The optimal conditions for producing hydrogen from sago starch by using PGK culture at 60°C were achieved when using gelatinized dry starch medium with an initial pH of 6.5 and an initial starch concentration of 2.5 g/l. The maximum hydrogen yield obtained under these conditions was 442.2 ml H_2/g-$starch_{added}$ (80% of the theoretical limit). Hydrogen yields were decreased at the lower and higher initial pH values, at higher initial starch concentrations, and when using non-gelatinized starch.

PCR-DGGE profiles of the 16S rRNA gene fragments from the cultures showed that the predominant species associated with efficient hydrogen production were closely related to the thermophilic anaerobic bacteria, including *T. saccharolyticum, T. thermosulfurigenes,* and the uncultured *Thermoanaerobacterium* sp.; these microbes may simultaneously produce hydrogen and ethanol. Thermophilic bacilli capable of utilizing starch, including *Bacillus* sp., *Anoxybacillus* sp., *B. lentus*, and *Geobacillus* sp., were also found in the cultures, but mainly in the non-gelatinized wet starch culture.

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