CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Characteristics of starch processing wastewater and seed sludge

Characteristics of the wastewater and seed sludge were displayed in the Table 4.1. Starch processing wastewater was suitable for fermentative hydrogen production due to high content of easy degradable carbohydrate that was consumed by microbes and function as their carbon source. Sen et al. (2012) used sago-starch wastewater as a substrate and produced hydrogen in range of 46.2 – 412.6 mL/g starch.

Table 4.1 Characteristics of seed sludge and starch processing wastewater

Parameters	Seed sludge	Starch processing wastewater	Unit
TS	36460.33	3042.13	mg/L
TSS	27166.33	1537.78	mg/L
VS	25843.33	2241.93	mg/L
VSS	19245.67	1507.67	mg/L
TKN	2426.70	308.00	mg/L
tCOD	1737.70	1967.20	mg/L
pH	7.36	4.31	-
Total iron	2.34	0.20	mg/L

4.2 Optimal environment for biohydrogen fermentation

In order to study an effect of the parameters on hydrogen yield, cumulative hydrogen production was plotted versus time and fitted with modified Gompertz equation (Lay et al., 1999) (Figure 4.1). The sigmoidal curves illustrate 3 main phases. The first phase before the first infection point, lag phase, displays low hydrogen

production because microbes need an adaptation into new environment (Zwittering et al., 1990). After that, microorganisms grow rapidly resulting to exponential increasing trend of cumulative hydrogen production after the first infection point. This phase is called exponential phase (Allaby, 2003). Slope in this phase is stated as a production rate (Zwittering et. al., 1990). After the second infection point, hydrogen gradually decreased production and eventually ceased due to shortage of nutrients resulting to lower the growth of microorganisms (Allaby, 2003).

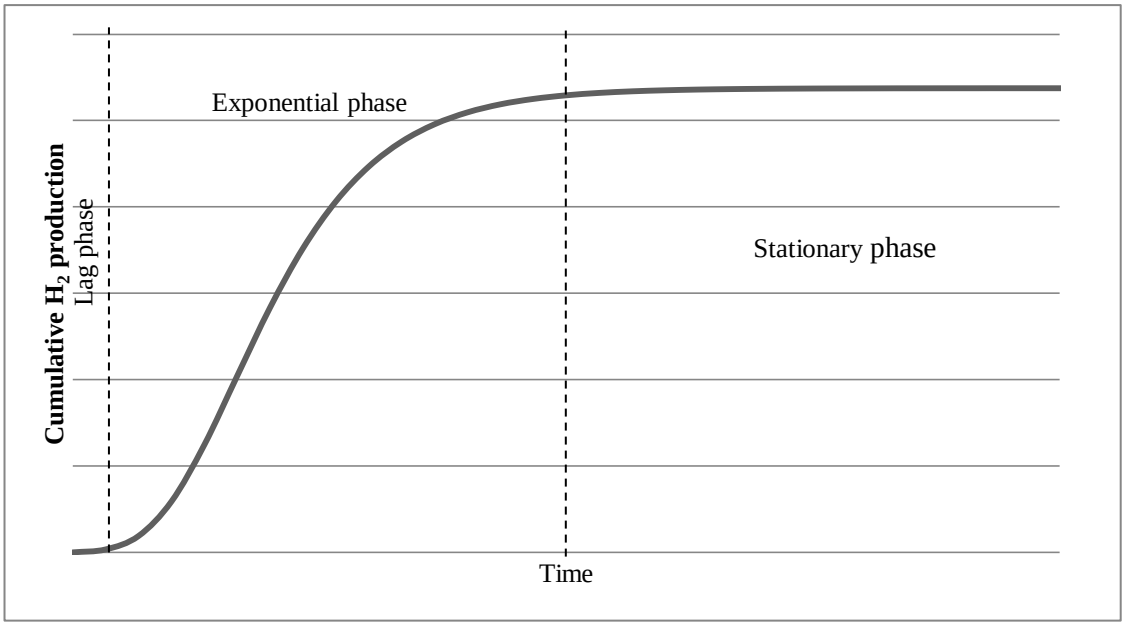


Figure 4.1 Example of Gompertz sigmoidal curve plotted between cumulative hydrogen production versus time

In this study, the fermentation spent around 7-14 days until no production of biogas was observed. The lag phase was about 8-12 hours. Lag period could display a period of time that microbes need to adapt into new environment (Khanna et al., 2011). The exponential phase was in range of 2-7 days. There was no hydrogen but only carbon dioxide observed in the stationery phase.

4.2.1 Effect of temperature and initial pH on biohydrogen production

At mesophilic temperature, total biogas was 794.65 – 864.65 mL containing hydrogen 1.77-3.20 %, carbon dioxide 24.81 – 32.92 %, and no methane

production observed (Table 4.2). Overall final pH decreased due to acid product generation (Wang and Wan, 2008b). Hydrogen yields at various initial pH were 13.42 mL/g COD (initial pH 4.0), 7.47 mL/g COD (initial pH 5.0), 9.52 mL/g COD (initial pH 6.0), 10.20 mL/g COD (initial pH 7.0), and maximum yield of 13.84 mL/g COD obtained at initial pH 8.0 (Table 4.3).

Table 4.2 Produced gas composition percentages at various temperature and initial pH

Temperature range	Initial pH	Gas composition (%)			
		H_2	CO_2	N_2	CH_4
Mesophilic	4	3.05	28.67	68.28	0
	5	1.77	24.81	73.42	0
	6	2.36	32.92	64.72	0
	7	2.41	31.53	66.06	0
	8	3.20	28.36	68.44	0
Thermophilic	4	6.32	47.25	46.43	0
	5	6.86	55.24	37.90	0
	6	6.11	53.48	40.41	0
	7	7.94	55.71	36.35	0
	8	7.02	53.29	39.69	0

Under thermophilic temperature, total biogas was 830.9 – 947.3 mL containing hydrogen 6.32 – 9.73 %, carbon dioxide 47.25 – 55.71 %, and no methane evolution detected (Table 4.2). Final pH also decreased from the initial value. Hydrogen yields at various initial pH were 28.37 mL/g COD (initial pH 4.0), 30.19 mL/g COD (initial pH 5.0), 25.81 mL/g COD (initial pH 6.0), 37.59 mL/g COD (initial pH 7.0), the maximum production, and 33.82 mL/g COD (initial pH 8.0) (Table4.3).

According to Table 4.2 and 4.3, it was found that higher temperature was able to enhance hydrogen production yield. The outcome was approximately 2-3 folded higher under thermophilic than mesophilic condition. VFA results were also higher in thermophilic than mesophilic range (Table 4.4). This may be an

inconsequence of lower solubility of hydrogen gas when increasing of temperature (Hawkes et al, 2002; Sivaramakrishna et al., 2014). Hawkes et al. (2002) reported that re-oxidation of reduced ferredoxin (Fd), which generating hydrogen, by microbes was less preferable when higher concentration of dissolved hydrogen. Moreover, thermophilic temperature could improve degradation of substrate (Sivaramakrishna et al., 2014).

Table 4.3 Hydrogen yield, hydrogen production, lag phase and final pH at various iron concentrations

Temperature range	Initial pH	H_2 yield (mL/gCOD)	H_2 production rate (mL/h)	Lag phase (h)	Final pH
Mesophilic	4	13.42	0.23	8	3.75
	5	7.47	0.14	8	4.75
	6	9.52	0.12	8	5.50
	7	10.20	0.35	10	6.00
	8	13.84	0.25	12	6.00
Thermophilic	4	28.37	0.31	8	3.50
	5	30.19	0.48	8	4.50
	6	25.81	0.30	8	5.75
	7	37.59	0.41	10	6.75
	8	33.82	0.93	12	7.50

Various initial pH values resulted differently effect to hydrogen yield. This study observed higher hydrogen yield in neutral and slightly alkaline initial pH than acidic conditions (Table 4.3). Maximum hydrogen yield was obtained at initial pH 8.0 (13.84 mL/g COD) and 7.0 (37.59 mL/g COD) under mesophilic and thermophilic temperature, respectively. Neutral and slightly basic conditions were more preferable for fermentative hydrogen production in this study.

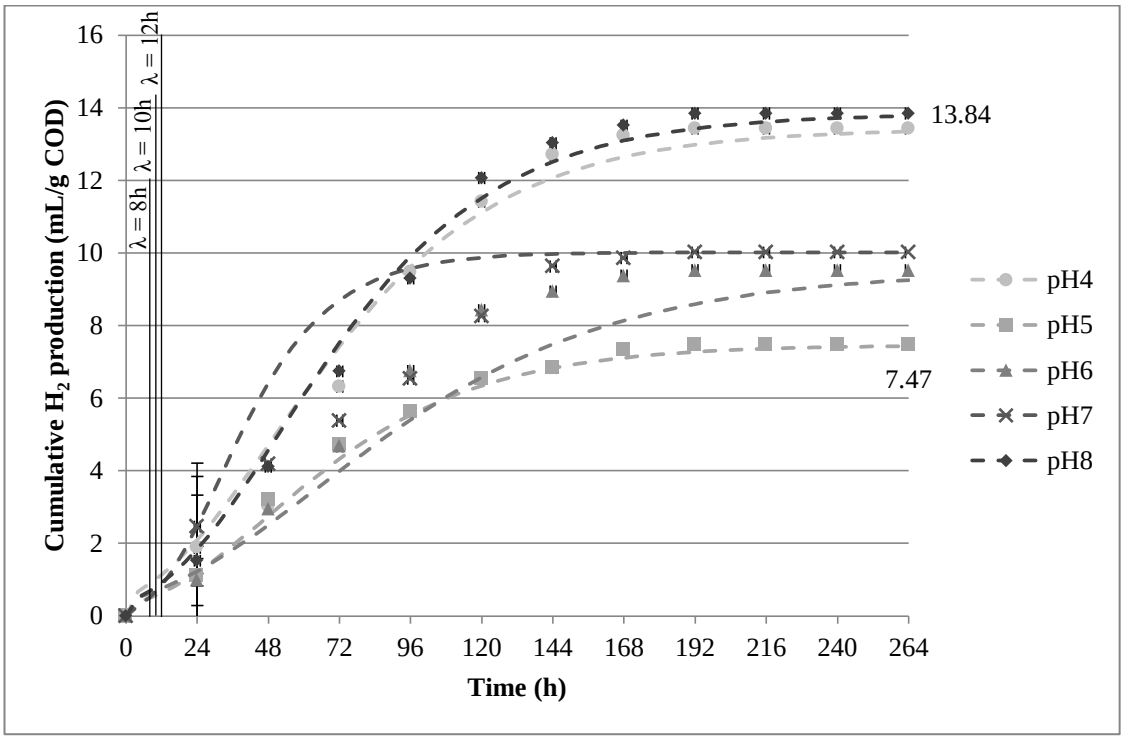


Figure 4.2 Cumulative hydrogen production plotted versus time at various initial pH under mesophilic temperature.

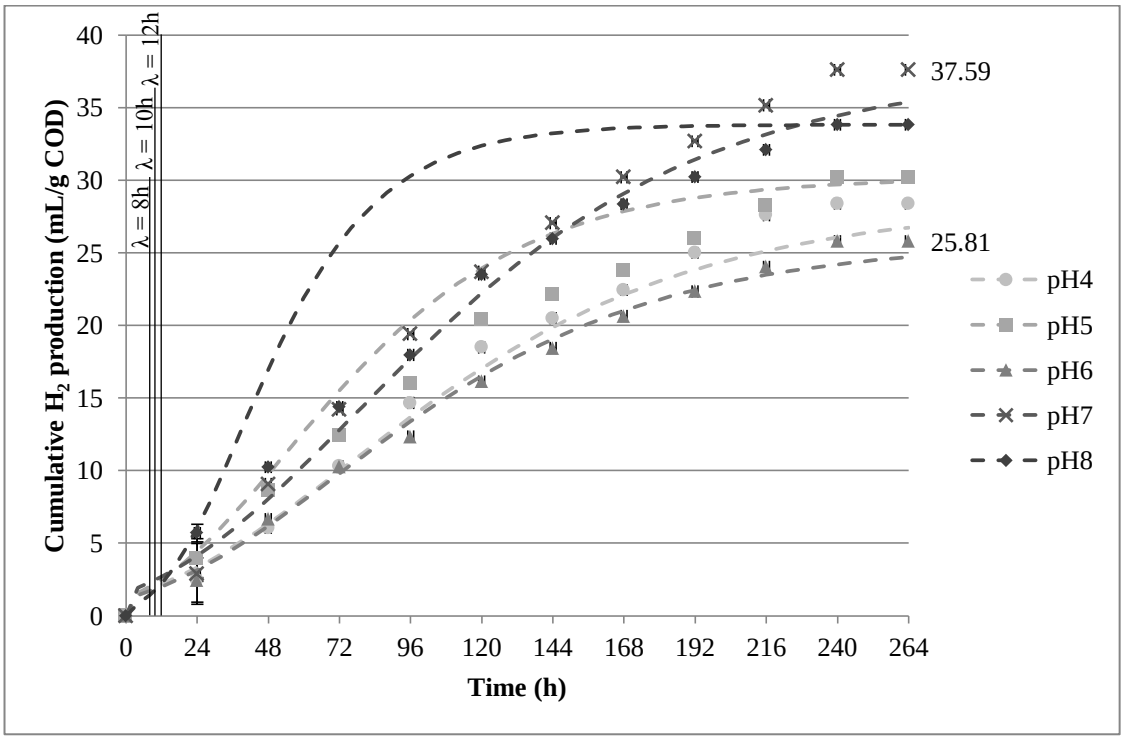


Figure 4.3 Cumulative hydrogen production plotted versus time at various initial pH under thermophilic temperature.

Table 4.4 VFA and COD removal percentage at various temperature and initial pH

Temperature range	Initial pH	H_2 yield (mL/gCOD)	VFA (ppm)			COD removal (%)
			HAc	HPr	HBu	
Mesophilic	4	13.42	6.53	2.79	0.00	23.05
	5	7.47	9.70	3.12	8.75	5.05
	6	9.52	19.6	1.65	11.35	17.05
	7	10.20	9.82	0.00	2.52	53.03
	8	13.84	6.60	0.00	1.17	56.03
Thermophilic	4	28.37	10.02	11.95	166.3	20.05
	5	30.19	16.63	19.69	91.8	17.05
	6	25.81	14.40	1.29	2.85	38.04
	7	37.59	7.28	0.00	1.00	14.05
	8	33.82	4.79	0.00	0.66	9.94

However, it was found that acetate (HAc) and butyrate (HBu) were generated higher in acidic than neutral and slightly basic condition, and no propionic acid (HPr) was observed at initial pH 7.0 and 8.0 condition (Table 4.4).

HPr generation reaction may cause hydrogen consumption (Eq 4.1) (Ghimire et al., 2015; Antonopoulou et al., 2008) hence less HPr production may result to more hydrogen yield. Under neutral or slightly basic initial pH condition, the possible hydrogen consumption was lower that acidic condition hence higher hydrogen yield.

$$C_6H_{12}O_6+2H_2 \rightarrow 2CH_3CH_2COOH+2H_2O \qquad \text{(Eq 4.1)}$$

Moreover, the substrate was possibly converted to acetate without hydrogen generation such as Eq 4.2 (Ghimire et al., 2015; Hawkes et al., 2007; Kim et al., 2006). According to many publications, hydrogen is generally produced through pathways that give acetic acid (HAc) and butyric acid (HBu) as by-products (Ghimire et al., 2015; Show et al., 2011), but it may be produced via other pathway, pyruvate-formate lyase (PFL), which generating formic acid (HCOOH) which can convert to

hydrogen and carbon dioxide (Eq 2.23 and 2.27) (Ntaikou et al., 2010). Lee (2009b) reported PFL pathway favors neutral pH.

$$2C_6H_{12}O_6 \rightarrow 3CH_3COOH+2CH_3CHOHCOOH \quad \text{(Eq 4.2)}$$

In addition, it was also found that initial pH influenced to the beginning of hydrogen evolution time (Table 4.3). Lag phase of 8 hours observed when initial pH 4.0-6.0 and increased to 12 hours at initial pH 8.0. Longer of lag time indicated that microbes need more time for adaptation into new environment (Khanna et al., 2011). Similar trend of increasing of lag phase from initial pH 6.0-8.0 was also observed in publications. Liu and Shen (2004) and Xiao et al. (2013) reported longer lag phase when increasing initial pH from 6.0 to 8.0 in anaerobic fermentation of starch and glucose, respectively.

Another advantage of fermentative biohydrogen production is to reduce COD of wastewaters which used as substrate. However, in some fermentation COD may be not largely decreased due to production of organic acid by-products. This can be solved by further fermentation for methane production (Antonopoulou et al 2008). Besides, carbon dioxide gas is able to further use in many ways for example, as a substrate to produce methanol and dimethyl ether (Olah et al., 2009).

Nevertheless, optimal initial pH and operational temperature for biohydrogen production were reported different values (Table 4.5). Sen and Sutra (2012) found that initial pH 7.0 is the most favorable for production of biohydrogen from Sago-starch wastewater by mixed culture under mesophilic temperature. Phowan and Danvirutai (2014) obtained highest hydrogen production from cassava pulp hydrolysate by mixed microbes at initial pH 5.5 operated under 35°C. Different optimal initial pH and temperature effect on biohydrogen production may primarily result from dissimilarity of dominant microbial species in mixed culture and their sources (Wang ang Wan, 2009). Besides, the dominant species may possibly deviate under different operational environments.

Table 4.5 Optimal initial pH and temperature in other studies

Substrate	Culture	Optimal initial pH	Optimal temperature (°C)	Study
Starch processing wastewater	Mixed	7.0	55±2	This study
Coconut juice wastewater	Mixed	5.0	35±2	Wongthanate et al. (2014)
Cassava pulp hydrolysate	Mixed	5.5	35	Phowan and Danvirutai (2014)
Sago-starch wastewater	Mixed	7.0	Mesophilic	Sen and Sutra (2012)
Sago-starch in wastewater	Mixed	6.5	Thermophilic	Hasyim et al. (2011)
Starch wastewater	Mixed	6.5	37	Wei et al. (2010)
Cassava starch	Mixed	6.0	37	Lee et al. (2008)

Consequently, optimal initial pH and temperature that gave highest hydrogen yield in this work were pH 7.0 and thermophilic condition. This condition was selected to further identify optimal iron concentration.

4.2.2 Effect of initial iron concentration

Total biogas was 1033.50 – 1212.25 mL composing of hydrogen 4.73 – 10.54 %, carbon dioxide 50.37 – 57.08 %, and no methane production observed (Table 4.6). Hydrogen yields at various iron concentration were 26.87 mL/g COD (0 mg/L), 55.39 mL/g COD (200 mg/L), 58.06 mL/g COD (400 mg/L), 56.70 mL/g COD (600 mg/L), 61.76 mL/g COD (800 mg/L), the maximum production, and 50.17 mL/g COD (1000 mg/L) (Table 4.7).

According to Figure 4.4 and Table 4.6, external addition of iron (Fe) obtained 2-3 folded higher of hydrogen yield than no iron addition. Hydrogen yield increased when increasing initial iron concentration from 0 to 400 mg/L, but slightly declined at 600 mg/L. Maximum hydrogen yield of 61.76 mL/g COD was found at initial iron concentration of 800 mg/L, and dropped at 1000 mg/L.

Table 4.6 Produced gas composition percentage at various initial iron concentrations

Initial Fe concentration (mg/L)	Gas composition (%)			
	H_2	CO_2	N_2	CH_4
0	4.73	50.37	44.91	0
200	10.54	51.83	37.62	0
400	9.97	57.08	32.95	0
600	9.60	56.06	34.34	0
800	10.02	52.87	37.11	0
1000	8.86	53.16	37.97	0

Since iron is a key component in hydrogenase enzyme which responsible for hydrogen generation. Increasing of iron can enhance hydrogenase activity and hydrogen production. However, too much iron may be toxic for hydrogen-producing bacteria. Many publications also reported decreasing of hydrogen yield when using initial iron concentration more than 1000 mg/L (Yang and Shen, 2006; Wang and Wan, 2008b; Zhang et al., 2005; Lee et al., 2001). Therefore, it may estimate that initial iron concentration of 1000 mg/L or more may be an iron toxicity limitation to hydrogen-producing microbes.

Besides, Lag phase observed 10 hours at all concentration which possibly meant iron addition did not affect the adaptation time of microbes in this experiment (Table 4.6).

The final pH was in range of 6.75-6.00 as initial iron concentration increasing. Wang and Wan (2008b) also observed decreasing of final pH when initial iron concentration increasing from 250-1500 mg/L. This was a result of the fermentation that generating acidic by-products. Besides, iron may affect a buffering capacity of the system since final pH was lower while initial iron concentration increasing. The buffer intensity affects an unchangeable of pH, the weaker buffer capacity, the lower pH (Lu and Lee, 2015; Zhu et al., 2008; Yang and Shen, 2006).

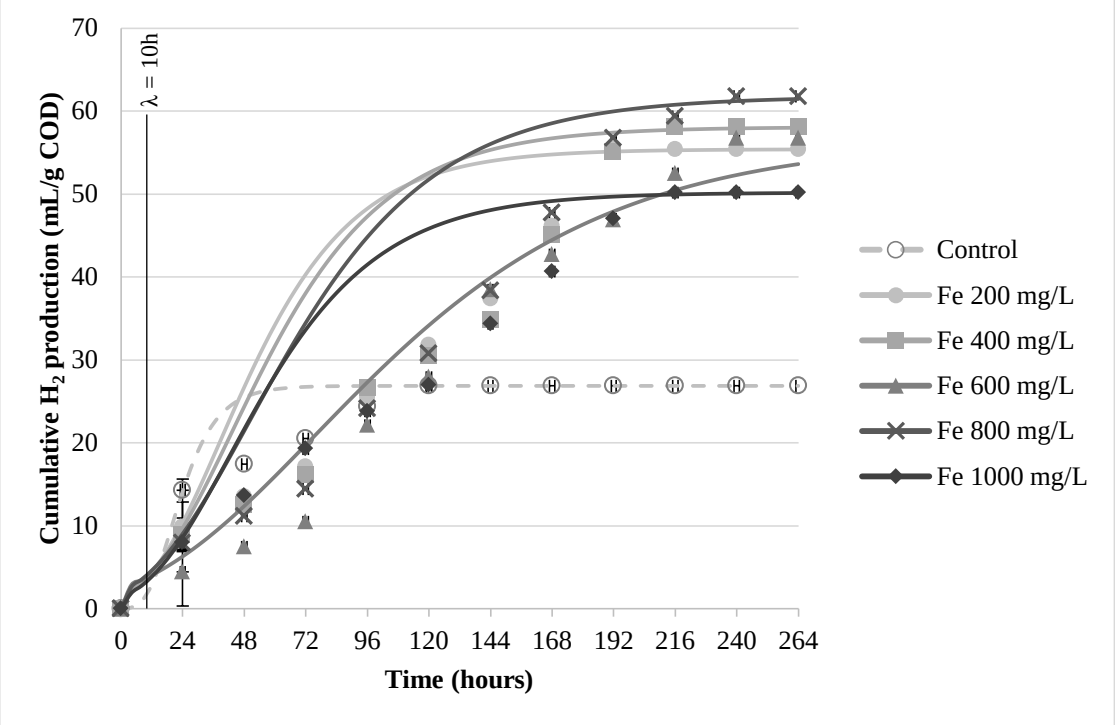


Figure 4.4 Cumulative hydrogen production plotted versus time at various initial iron concentrations

Table 4.7 Hydrogen yield and production rate, lag phase and final pH at various iron concentrations

Iron concentration (mg/L)	H_2 yield (mL/gCOD)	H_2 production rate (mL/h)	Lag phase (h)	Final pH
0	26.87	2.01	10	6.75
200	55.39	1.39	10	6.75
400	58.06	1.26	10	6.50
600	56.70	0.63	10	6.00
800	61.76	1.11	10	6.00
1000	50.17	1.12	10	6.00

VFA were also generated much higher than no iron insertion resulting to greater hydrogen yield (Table 4.8). However, at maximum production condition (initial iron concentration 800 mg/L), HAc and HBu were observed lower than initial

iron concentration 200-600 mg/L condition, but HPr was also observed lower. At initial iron 200-600 mg/L, HPr was higher than initial iron concentration 800 mg/L environment which may give lower results of hydrogen generation.

Table 4.8 VFA and COD removal percentage at various initial iron concentrations

Initial Fe concentration (mg/L)	H_2 yield (mL/gCOD)	VFA (ppm)			COD removal (%)
		HAc	HPr	HBu	
0	26.87	5.40	0	0.52	17.05
200	55.39	199.90	88.56	61.49	3.94
400	58.06	100.19	37.73	25.93	21.93
600	56.70	76.25	25.79	19.05	50.03
800	61.76	51.95	14.22	9.34	80.01
1000	50.17	33.42	7.74	5.12	38.04

Many publications informed increasing of hydrogen yield when adding proper amount of iron but reported different optimal initial iron concentration (Table 4.9). Some works reported highest hydrogen production at initial iron concentration lower than 100 mg/L (Yang et al., 2006; Zhang and Shen. 2006), while some studies obtained maximum yield at initial iron concentration higher than 300 mg/L (Wang and Wan, 2008b; Zhang et al., 2005). Possible reasons may come from different substrate, source of microbes and/or dominant species, operated conditions. Therefore, optimal iron concentration should be studied hinging on situation.

In this study, optimal initial iron concentration that produced maximum hydrogen yield in present study was 800 mg/L. This value was used in further experiments together with optimal initial pH 7.0 and thermophilic temperature.

Table 4.9 Optimal initial iron concentration from various publications

Substrate	Culture	Optimal [Fe^{2+}] (mg/L)	Study
Starch processing wastewater	Mixed	800	This study
Soluble starch	Mixed	55.3	Yang et al. (2006)
Sucrose	Mixed	73.47	Zhang and Shen (2006)
Sucrose	Mixed	300	Wang and Wan (2008b)
Sucrose	Mixed	587.76	Zhang et al. (2005)

4.3 Biomaterials (BM) characterization and selection

All BM, coir (CO), corncob (CC), loofa sponge (LS), pine tree bark (PT), silk cocoon (SC), shell (SH), and crab exoskeleton (CE), were tested for acid tolerance and characterized for average density. Then they were investigated for cell immobilization ability and hydrogen production efficiency. BM which gave the best positive results from each group were further analyzed its surface area and used to identify optimal BM concentration for hydrogen production. The result displays in Table 4.10.

4.3.1 Acid tolerance

All BM from plants were able to tolerate in acid environment, while from animal, only silk cocoon and shell could endure acidity (Table 4.10 and Figure 4.5). Crab exoskeleton became soften and some pieces were broken into smaller pieces. Besides, there was also observed white substances in the aqueous after the experiment (Figure 4.5 (g)). These white substances may be protein matrixes that cover the chitin fiber (Figure 2.15). Proteins are able to denature by pH variation which can adjust the ionization states of amino acids in the molecules (Voet and Voet, 2004). Hence, crab shell was not selected to test in further experiment.

4.3.2 Hydrogen production efficiency and cell immobilization ability

Hydrogen production efficiency of each BM was studied at the BM concentration of 5% (v/v). The yields were in range of 13.49 - 19.42 mL/g COD, and rose as immobilization ability increases (Table 4.10).

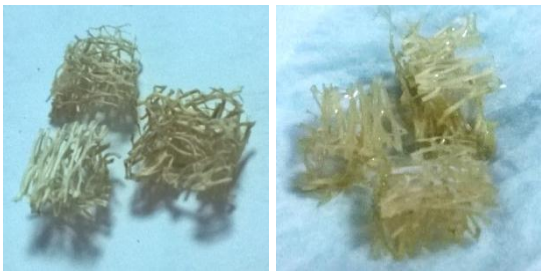
Among BM from plants, maximum production was 19.42 mL/g COD when using loofa sponge (0.080 mg/g BM of cell immobilization ability), following by 18.97 mL/g COD (CO, 0.066 mg/g BM), 17.63 mL/g COD (PT, 0.058 mg/g BM), and 12.92 mL/g COD (CC, 0.019 mg/g BM), respectively. In BM from animal, the fermentation with SC (19.41 mL/g COD) was found better hydrogen yield than SH (13.49 mL/g COD). Also, the cell immobilization ability of SC (0.031 mg/g BM) was larger than SH (0.013 mg/g BM).

Accordingly, loofa sponge was obtain the highest hydrogen yield (19.42 mL/g COD) among BM from plants while cocoon helped generating the greatest amount of hydrogen (19.41 mL/g COD) among BM from animals. Also, LS and SC were observed highest cell immobilization ability among its group which probably meant microorganisms preferable to growth on them. However, LS and SC obtained similar amount of hydrogen production while different in cell immobilization ability, 0.080 and 0.031 mg/g BM, respectively.

Nevertheless, loofa sponge and cocoon were selected to study for its optimal usage concentration in the next step as a representative of their group.

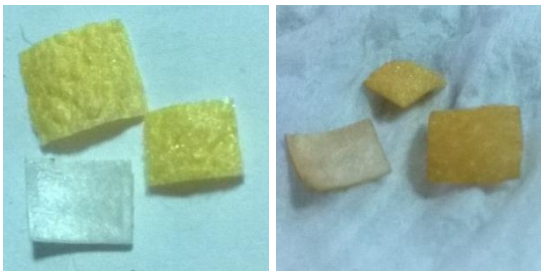
(a) Coir (CO)

(b) Corncob (CC)

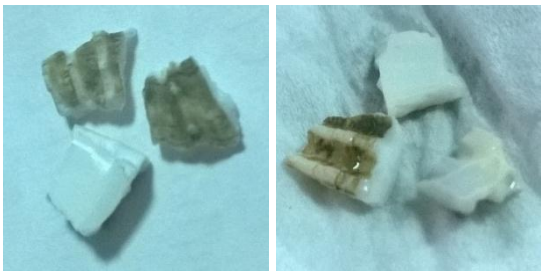


(c) Loofa sponge (LS)

(d) Pine tree bark (PT)



(e) Silk cocoon (SK)



(f) Shell (SH)

(g) Crab exoskeleton (CE)

Figure 4.5 (a-f) Each BM before (left) and after (right) (g) CE before (left) and after (middle and right) suspended in acetic acid pH 4.0 for 7 days

Table 4.10 Physical characterization, cell immobilization ability, and hydrogen production efficiency of BM

BM		Acid tolerance	Average density (g/cm^3)	Surface area (m^2/g)	Cell immobilization (mg/g BM)	H_2 yield (mL/gCOD)
Plant	CO	Yes	0.098	-	0.066	18.97
	CC	Yes	0.458	-	0.019	12.92
	LS	Yes	0.045	174.50	0.080	19.42
	PT	Yes	0.335	-	0.058	17.63
Animal	SC	Yes	0.190	326.40	0.031	19.41
	SH	Yes	1.471	-	0.013	13.49
	CS	No	-	-	-	-

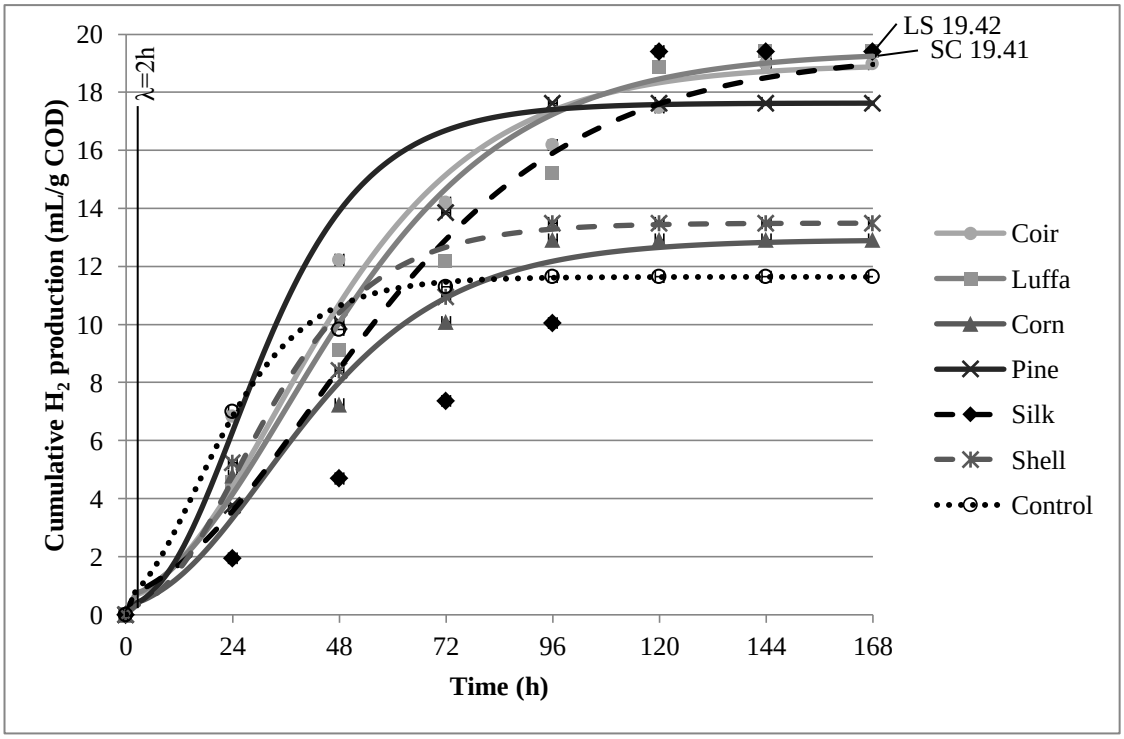


Figure 4.6 Cumulative hydrogen production plotted versus time with addition of various BM

4.4 Optimal concentration of BM for biohydrogen production

Loofa sponge and silk cocoon were identified for optimal BM concentration from 0 - 20% (v/v). Lag phase of control condition (0%) was longer than conditions that containing BM. Since BMs could enhance a response of bacterial reproducibility (Michelini and Roda, 2012), the microbes could adapt into new environment easier and hence shorter lag period.

When using LS, total biogas was 662.25 – 710.00 mL composing of hydrogen 2.79 – 7.44 %, carbon dioxide 36.23 – 43.40 %, and no methane production observed. Hydrogen yields at various BM concentration were 11.64 mL/g COD (0 %), 26.43 mL/g COD (5 %), 21.22 mL/g COD (10 %), 11.08 mL/g COD (15 %), and 9.66 mL/g COD (20 %) (Table 4.11).

In case of SC, produced biogas was 851.80 – 977.30 mL containing of hydrogen 1.11 – 5.76 %, carbon dioxide 40.11 – 44.26 %, and methane generation did not found. Hydrogen evolution at various BM concentration was 24.12 mL/g COD (0 %), 24.26 mL/g COD (5 %), 17.94 mL/g COD (10 %), 7.50 mL/g COD (15 %), and 5.18 mL/g COD (20 %) (Table 4.12).

Accordingly, addition of BM could enhance hydrogen yield. Maximum hydrogen production of 26.43 and 24.26 mL/gCOD was observed at 5% (v/v) of BM in plant and animal-based BM, respectively. However, in SC, there was not much difference between the control (0% BM) and 5% (v/v) BM insertion. Furthemore, hydrogen yield decreased when increasing of BM concentration which may cause from restriction of flow area in the fermentation system (Klebanoff, 2012) and/or decreasing of void space for bacterial growth (Li and Yu, 2011, pp. 544-545).

VFA results was also found greater concentration of VFA when insertion of BM than 0% (v/v) BM. Even though, VFA was produced larger with BM concentration increasing, above 5% (v/v) HPr was also generated greater that may reduce hydrogen yield.

Lag phase was also observed shorter than previous step which meant microbial spend lesser time to adopt into new environment. This possibly resulted from applying supporting materials. Zhao et al. (2012) reported whole cell immobilization could enhance the activity of microbes.

Table 4.11 Hydrogen yield, hydrogen production, lag phase and final pH at various loofa sponge concentrations

Concentration (%)	H_2 yield (mL/gCOD)	H_2 production rate (mL/h)	Lag phase (h)	Final pH
0	11.64	0.86	10	5.00
5	26.43	1.70	8	5.00
10	21.22	1.27	8	5.50
15	11.08	0.83	8	5.50
20	9.66	0.70	8	5.00

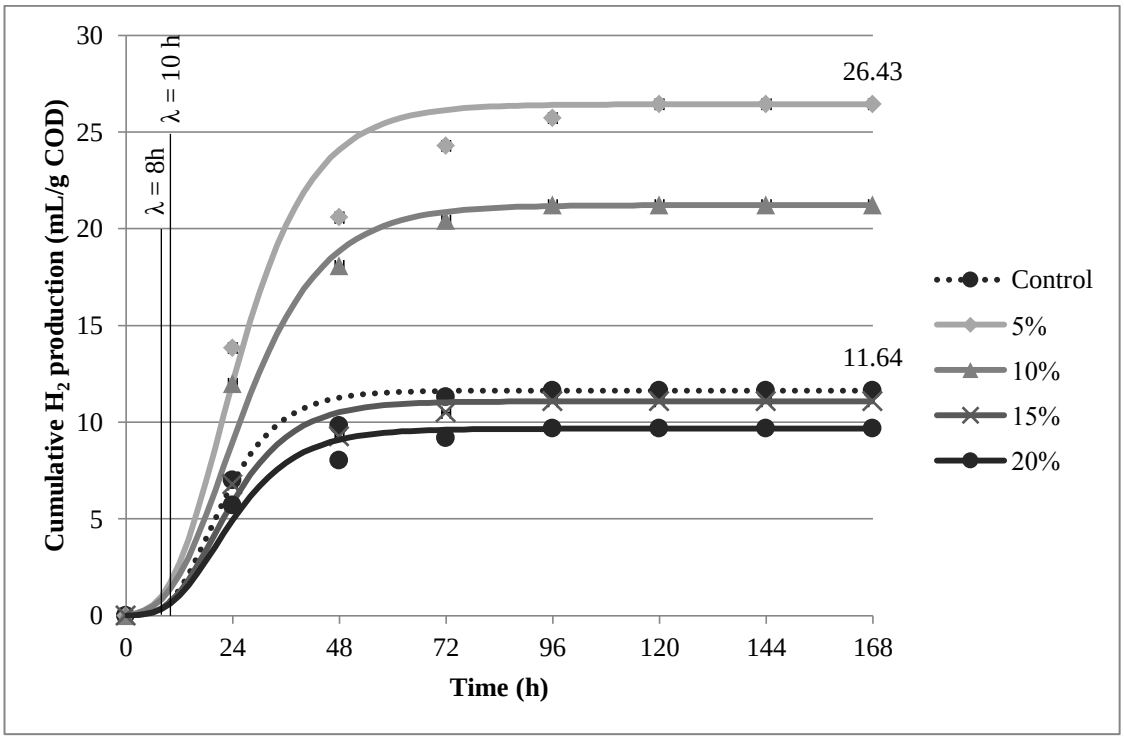


Figure 4.7 Cumulative hydrogen production plotted versus time at various BM (loofa sponge) concentration

Table 4.12 Hydrogen yield, hydrogen production, lag phase and final pH at various cocoon concentrations

Concentration (%)	H_2 yield (mL/gCOD)	H_2 production rate (mL/h)	Lag phase (h)	Final pH
0	24.12	2.32	10	6.75
5	24.26	2.98	8	6.25
10	17.94	1.67	8	6.50
15	7.50	0.92	8	6.50
20	5.18	0.64	8	6.50

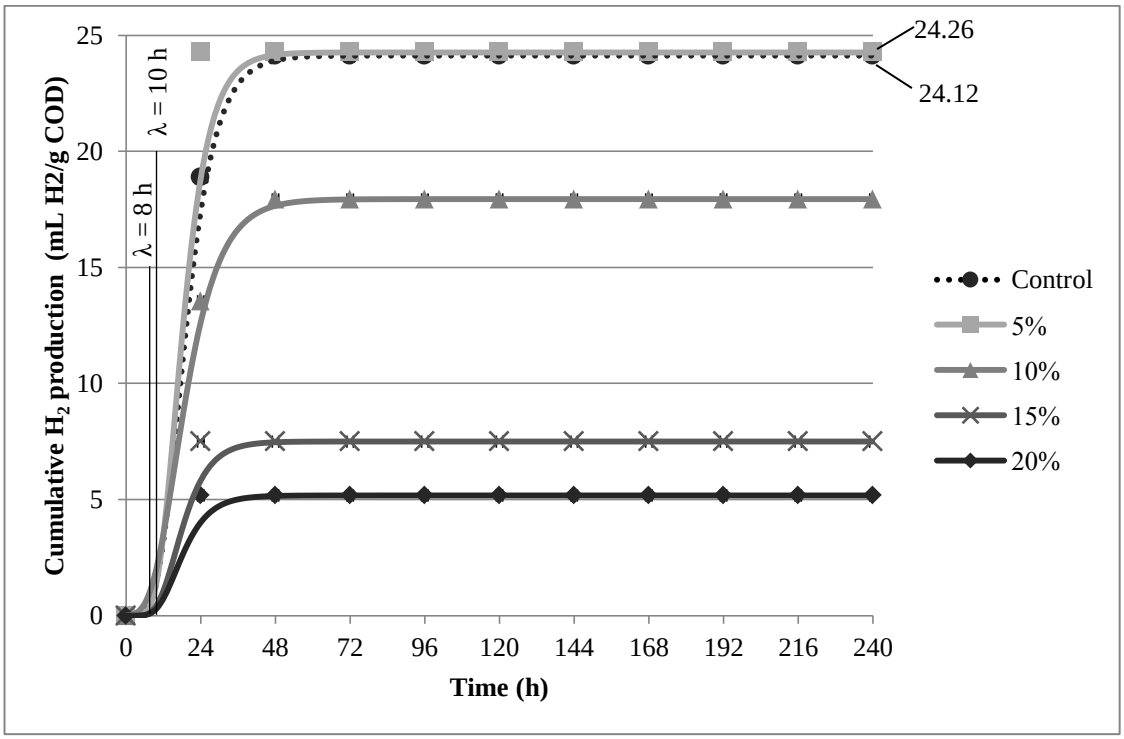


Figure 4.8 Cumulative hydrogen production plotted versus time at various BM (cocoon) concentration

Table 4.13 VFA and COD removal percentage at various loofa sponge concentrations

Concentration (%)	H_2 yield (mL/gCOD)	VFA (ppm)			COD removal (%)
		HAc	HPr	HBu	
0	11.64	51.34	28.00	171.46	76.68
5	26.43	63.61	35.62	301.30	66.69
10	21.22	44.93	35.40	175.95	66.69
15	11.08	88.57	52.20	334.55	16.72
20	9.66	103.62	42.73	293.96	3.27

Table 4.14 VFA and COD removal percentage at various cocoon concentrations

Concentration (%)	H_2 yield (mL/gCOD)	VFA (ppm)			COD removal (%)
		HAc	HPr	HBu	
0	24.12	8.76	12.93	48.09	46.70
5	24.26	12.87	17.74	67.26	56.70
10	17.94	26.09	36.85	116.14	3.27
15	7.50	38.93	55.42	143.67	29.92
20	5.18	67.55	93.98	198.79	60.02

There were studies that reported enhancement of biogas and/or hydrogen production with applying immobilized supporting materials. Zheng et al. (2008) observed increasing of yield from range of 0.4-1.7 mol H_2/mol glucose to maximum yield of 7.6 mol H_2/mol glucose. Szentgyorgyi et al. (2010) reported biogas generation increasing 28% due to cell immobilization.

However, amount of supporting materials was varied depending on type of the materials and results were different on account of substrate, reactor type, and so on (Table 4.15).

Nevertheless, optimal concentration of LS (plant) and SC (animal) for hydrogen production in this study were both 5% (v/v). This value was used to compare and identify which BM from plant or animal was better to enhance the hydrogen generation from starch processing wastewater by anaerobic mixed culture in the next step.

Table 4.15 Amount and type of supporting materials and hydrogen production from various publications

Substrates	Culture	Reactor type	Supporting materials (SM)	SM amount in reactor	H_2 production	Study
Starch processing wastewater	Mixed	Batch	Loofa sponge	5 %(v/v)	24.63 mL/g COD	This study
Glucose	Mixed	Continuous	Expanded clay	24 %(v/v)	2.49 mol H_2/ mol glucose	Amorim at al. (2009)
Glucose	Mixed	Continuous	Expanded clay	Not mentioned	2.59 mol H_2/ mol glucose	Barros et al. (2010)
Glucose	Mixed	Continuous	Plastic carriers	90 %(v/v)	0.69 mol H_2/ mol glucose	Zheng et al. (2008)

4.5 Comparison BM from plants and animals

LS and SC, as a BM from plants and animal, at 5% BM concentration were repeatedly compared for its hydrogen production enhancement. For LS, total biogas was 935.50 containing of hydrogen 5.89% and carbon dioxide 38.87 %, while in case of SC, total biogas was 899.50 mL composing of hydrogen 3.88 % and carbon dioxide 44.60 %. Both BM did not obtain methane production. Lag phase was 8 hours as same as previous experiment, and final pH decreased to 6.5 from the initial value. Regarding Figure 4.9, when adding loofa sponge hydrogen yield was 28.03 mL/g COD and evolution rate was 2.68 mL/h. In contrast, hydrogen yield was 17.74 mL/g COD and generation rate was 1.42 mL/h when using cocoon.

Therefore, at their optimal concentration, loofa sponge obtained higher hydrogen yield and rate than cocoon. VFA result was also observed lower HPr generation in loofa condition which may reduce consumption of hydrogen.

According to Table 4.10 previously, total surface area of LS and SC at 5% (v/v) could be calculated and they were 70.43 and 558.14 m^2, respectively. The cell immobilization of LS and SC was 0.080 and 0.031 mg/g BM, respectively. This result showed that loofa sponge had lower total surface area but higher cell immobilization and hydrogen production yield. The higher cell immobilization may cause from larger pore diameter of loofa sponge (25.290 °A). (Pore diameter of cocoon was 18.710 °A). Li and Yu (2011) reported that higher porosity spaced void for growth of microorganisms. Thereby, microbes possibly preferred growing on loofa sponge which gave higher hydrogen production and rate. In addition, high surface area may not result to high cell immobilization and hydrogen production, if there was low void space for the growth of microorganisms. Thus, the surface area, pore diameter and cell immobilization should be considered together.

Consequently, BM from plants (loofa) was able to improve hydrogen production than BM from animals (cocoon).

Table 4.16 VFA and COD removal percentage of loofa and cocoon at its optimal concentration

BM	H_2 yield (mL/gCOD)	VFA (ppm)			COD removal (%)
		HAc	HPr	HBu	
Plant (loofa)	28.03	16.07	22.92	50.38	60.02
Animal (cocoon)	17.74	17.67	26.19	53.16	30.04

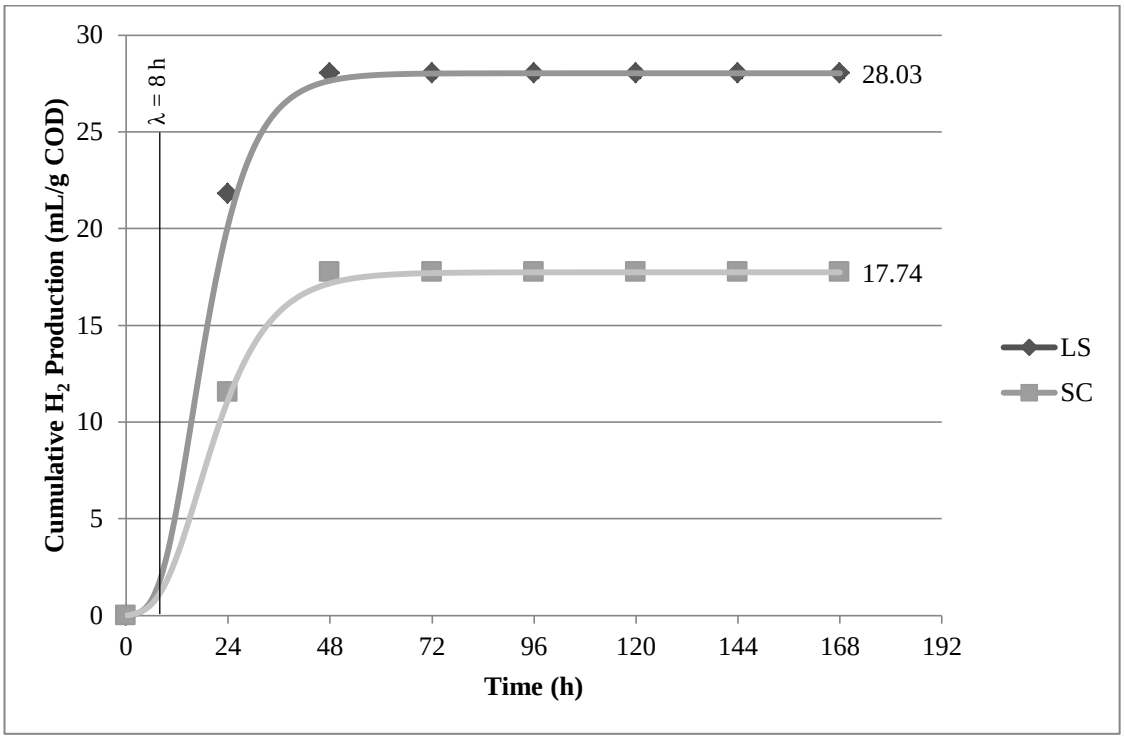


Figure 4.9 Cumulative hydrogen production plotted versus time of loofa and cocoon at its optimal concentration

4.6 Population and Morphology of microorganisms on BM

Loofa sponge and cocoon (5% v/v) was observed for bacterial population on their surface by bioinformatics analysis. Microbial population of the culture under optimal fermentation condition was analyzed by bioinformatics analysis. The DNA samples were clustered into OTU (Operational Taxonomic Units) at 97% similarity. Taxonomic ranks were assigned to OTU representative sequence using Ribosomal Database Project (RDP) Na,e Bayesian Classifier v.2.2. OTU number was 104 which primarily represent the degree of sample diversity. OTU rank abundance curve (Figure 4.13) provides an information about species richness and species evenness. Species richness can be viewed as the number of different species on the chart (X-axis), and species evenness is derived from the slope of the line that fits the graph. A steep gradient indicates low evenness as the high ranking species have much higher abundances than the low ranking species.

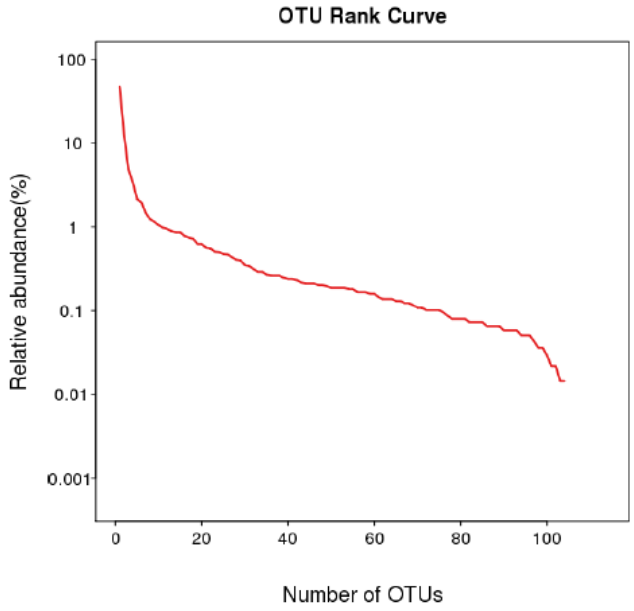
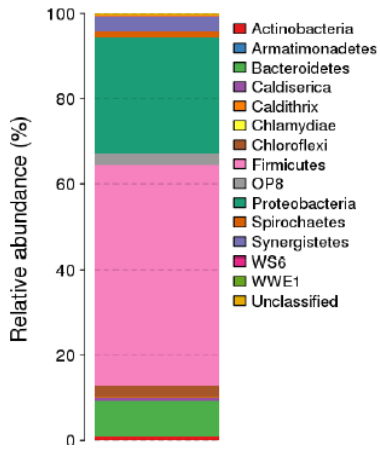


Figure 4.10 OTU rank curve

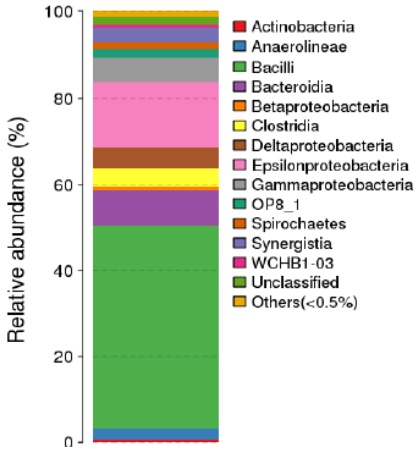
Moreover, the DNA samples were summarized based on taxonomic rank (Phylum, Class, Order, Family, Genus, and Specie) in a histogram (Figure 4.14 and 4.15). It was found that under optimal fermentation condition, dominant species was in Firmicutes phylum, Bacilli class, Bacillales order, Bacillaceae family, *Bacillus cereus* (*B. cereus*) with population around 47% of the mixed culture.

B. cereus is gram-positve, facultatively anaerobic, spore-formimg, and rod-shape microbes (Bottone, 2010). Zhang et al. (2014) reported that *B. cereus* is a hydrogen producer which efficiently using substrate such as starch and starch wastewater to produce hydrogen. B. cereus is found in soil and growing plants. It is able to endure severe environments by forming endospores which resisting to heat, dehydration and other physical stresses (Arnesen et al., 2008). B. cereus can cause diarrhoeal and emetic syndromes (Arnesen et al., 2008; Ivanova et al., 2003).

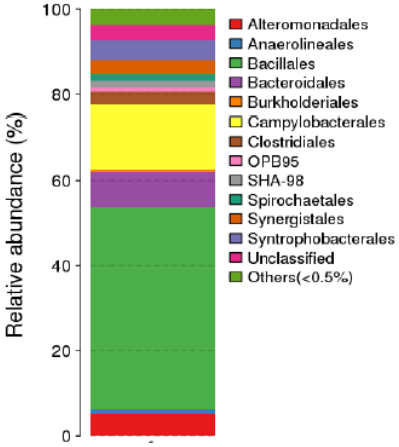
Microbial morphology was analyzed using SEM and FISH. Results of SEM image are illustrated as in Figure 4.10 and 4.11. Microorganisms that found on surface of the BM were mostly rod shape which also confirmed the result of bioinformatics analysis.



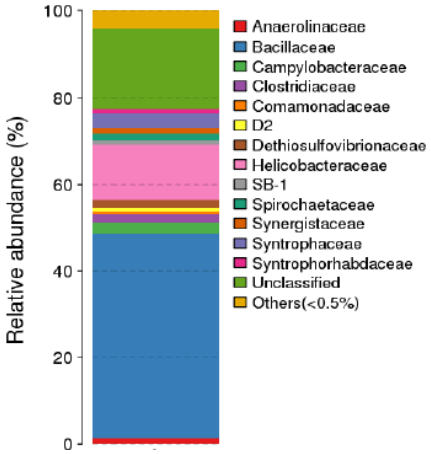
(a) Phylum-level



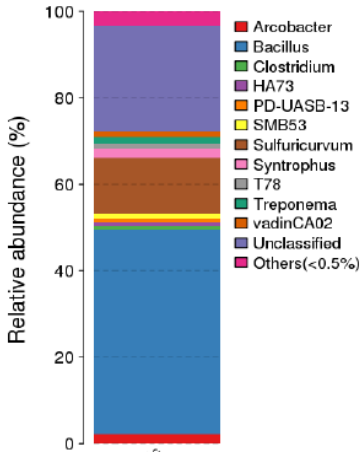
(b) Class-level



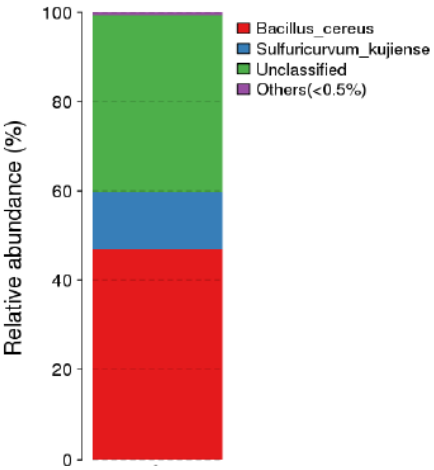
(c) Order-level



(d) Family-level



(e) Genus-level



(g) Specie-level

Figure 4.11 The taxonomic composition distribution in samples of (a) phylum-level (b) class-level (c) order-level (d) family-level (e) genus-level (f) species-level

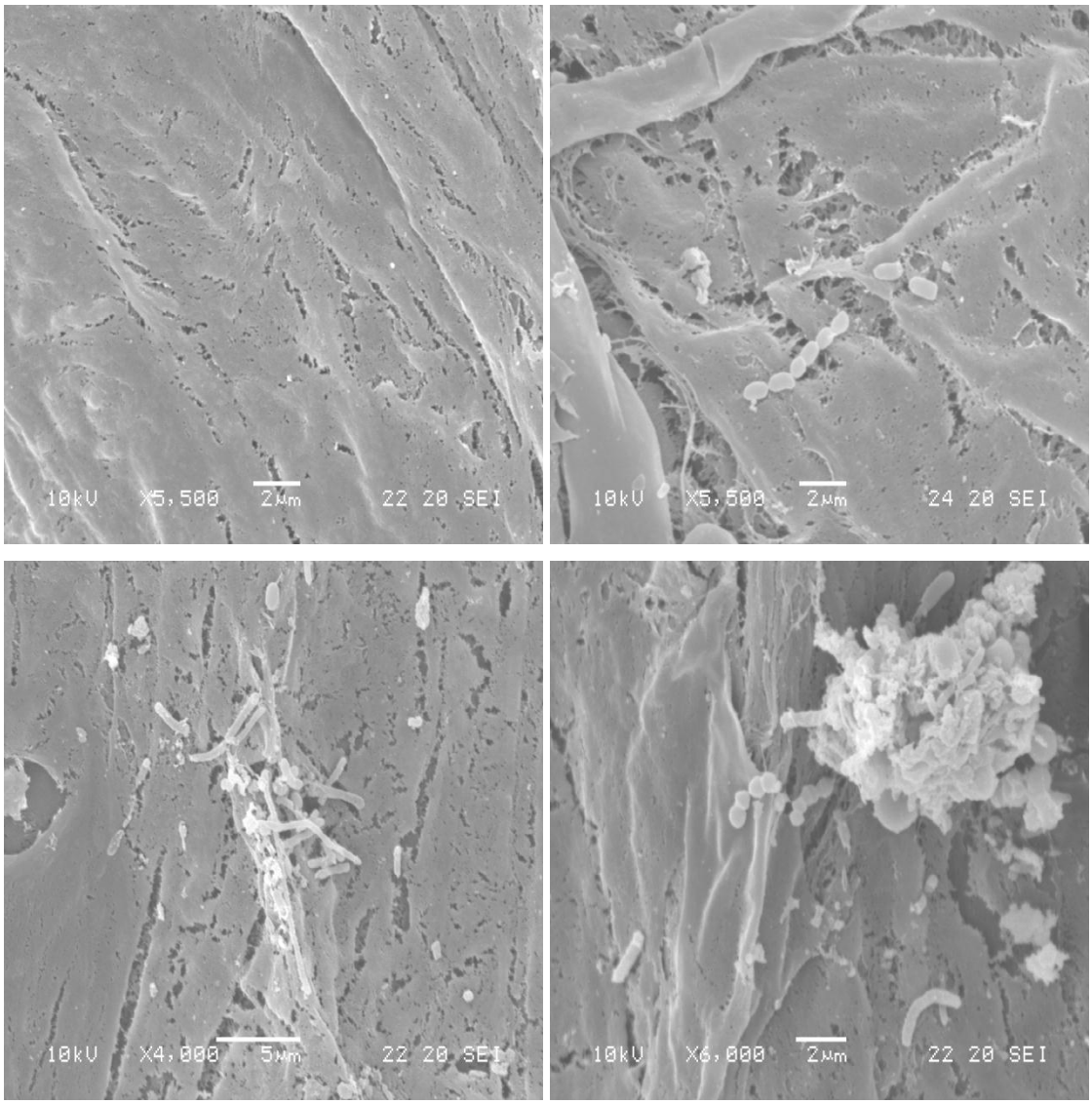


Figure 4.12 SEM image of loofa sponge before (top left) and after the fermentation

Fluorescence images (Figure 4.12) also confirmed observation of the same shape and range size of microbes. There are fluorescence images of (a) pure Bacillus as a positive result control, (b) microbes from the fermentation with addition of optimal concentration of loofa sponge and (c) silk cocoon. Cells lightened in blue were all microorganisms in the fermentation system, while the red fluorescence cells were specified with the probe pB196 labeled for Firmicutes phylum. Cells that indicated both blue and red color on previous two images would illustrate purple or violet in the right image. However, intensity of detected cells was different. This was possibly because of low RNA content in the cell. Metabolically less active or inactive cells may

contain low RNA content than the active one since they are in the rest state or lowering their reproduction (Kubota et al., 2006; Lebaron et al. 2001; Gasol et al., 1999).

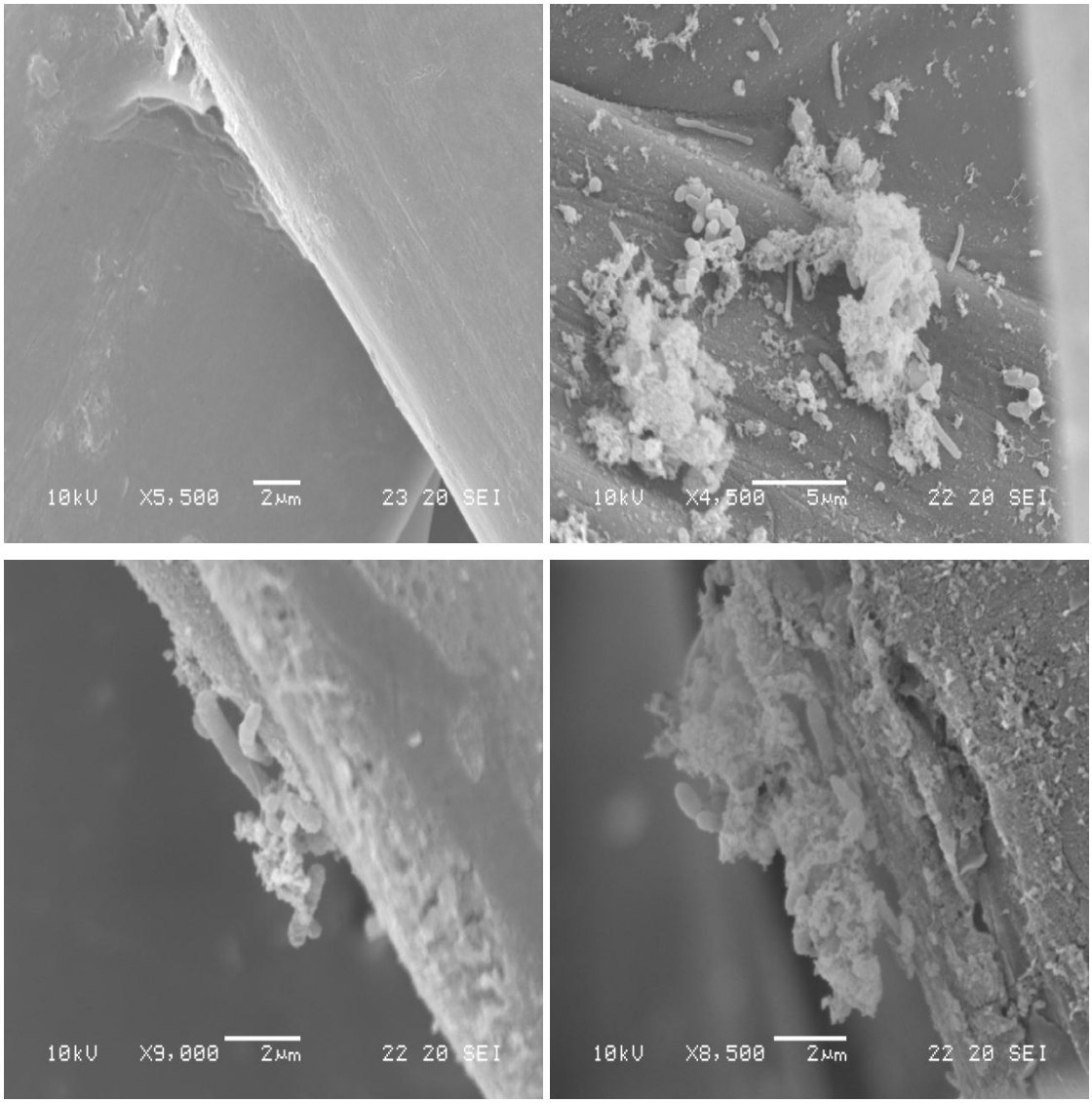
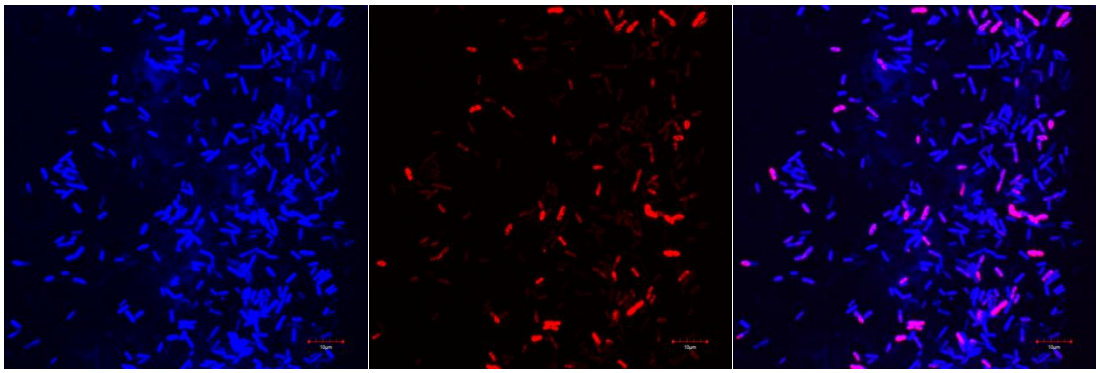
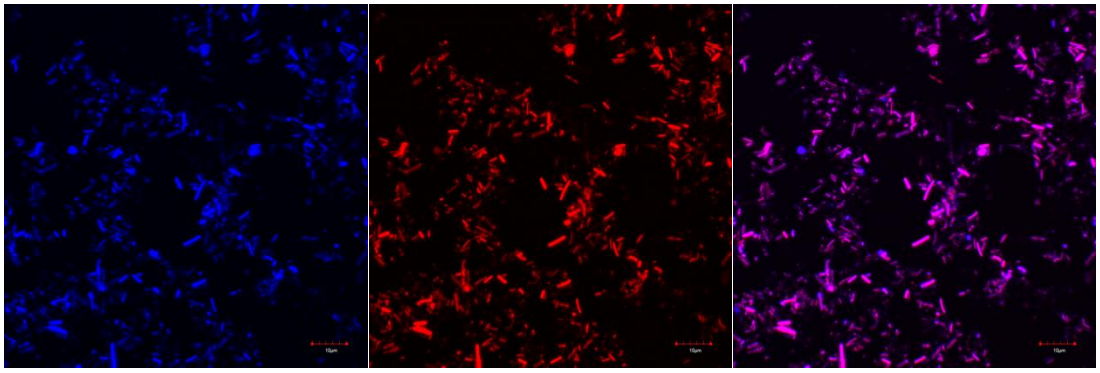


Figure 4.13 SEM image of cocoon before (top left) and after the fermentation

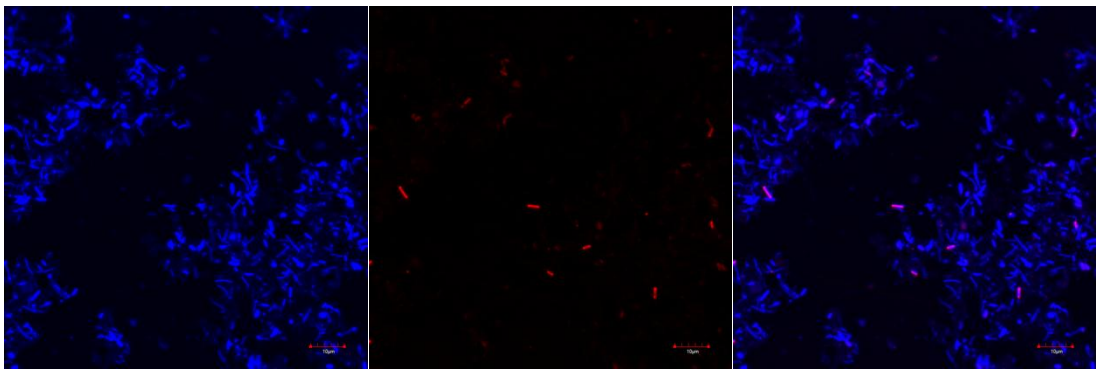
Accordingly, seeing that the probe for FISH analysis was specific to bacteria in Firmicutes phylum and many of them were detected by the probe, it would probably mean our hydrogen-producing microorganisms were in this phylum.



a. Control



b. LF 5% (v/v)



c. SC 5% (v/v)

Figure 4.14 Fluorescence images (magnified 120X) of (a) pure Bacillus (positive control) and (b)-(c) microorganisms from the surface of various BM