CHAPTER 4 RESULTS AND DISCUSSIONS

4.1Characteristics of Microbial Seeds

The biosludges were governed from different sources and they were from Dindang (DD) wastewater treatment plant and Nongkheam (NK) sewage wastewater treatment plant, Bangkok, Thailand. They were initially determined for basic characteristics and the concerned parameters were Suspended Solids (SS), Volatile Suspended Solids (VSS), Chemical Oxygen Demand (COD) and pH. The characteristics of microbial seed samples used in this study are presented in Table 4.1. The SS content of NK sludge was higher than DD sludge. The ratio of VSS to SS was determined to describe the percentage of biomass (organic component) in the sludge. This ratio indicated that both sludge contained a significant amount of biomass. The living biomass of both DD and NK sludges were familiar with the neutral pH, which were 6.63 and 7.35, respectively. These sludge samples were required sometimes to adapt themselves to tolerate with acidic pH of 5.5, which was controlled in biohydrogen process. The soluble COD (sCOD) presented in these wasted sludges were relatively low concentration. They may be acknowledged as the active biomass, which could effectively consume the organic residues in the domestic wastewater.

Table 4.1 Characteristics of microbial seed samples

Parameter	Microbial seed sample	
	Dindang wastewater treatment plant (DD)	**Nongkheam sewage wastewater treatment plant (NK)**
SS (g/L)	19.9	29.1
VSS (g/L)	9.80	12.77
VSS/SS	0.49	0.44
pH	6.63	7.35
sCOD (mg/L)	118	140

4.2 Series 1: Microbial Acclimatisation and Hydrogen gas Production Performance

4.2.1 Heat treated inocola

Set I: Bioreactor 1A-4A was fed with pure glucose and pure starch solutions

After the incubating period of 55 days, the treated DD and NK inocula were matured. The amounts of accumulation H_2 were 1264 and 792 mLH_2, when the DD and NK inocula were fed with glucose, respectively. If starch solution was supplied to DD and NK inocula, the accumulation H_2 gas were declined to 2.42 and 0 mLH_2, respectively. The heat treatment process may eliminate the microbes that could produce the cellulolytic enzymes, which could be able to hydrolyse the cellulose chains of starch. Yield of H_2 produced by direct fermentation of cellulolytic microbes in DD sludge is very low. The cumulative H_2 production during incubation period is presented in Figure 4.1. The hydrogen producing bacteria could resist the heat and could survive in acidic solution, however the hydrogen producing bacteria had a very slow biomass producing rate and less competitive ability than other acid forming bacteria. Hence, at the initial state, the natural microbial seeds were heat shocked to eliminate the other competitive microbes. The heat shock inoculums were then acclimatised with glucose, which was a basic necessity for microbes. The parameters including sCOD removal, VSS, SS and VSS to SS ratio of each type of inoculums were determined along the cultivation time. The raw data of this section is shown in Appendix A.

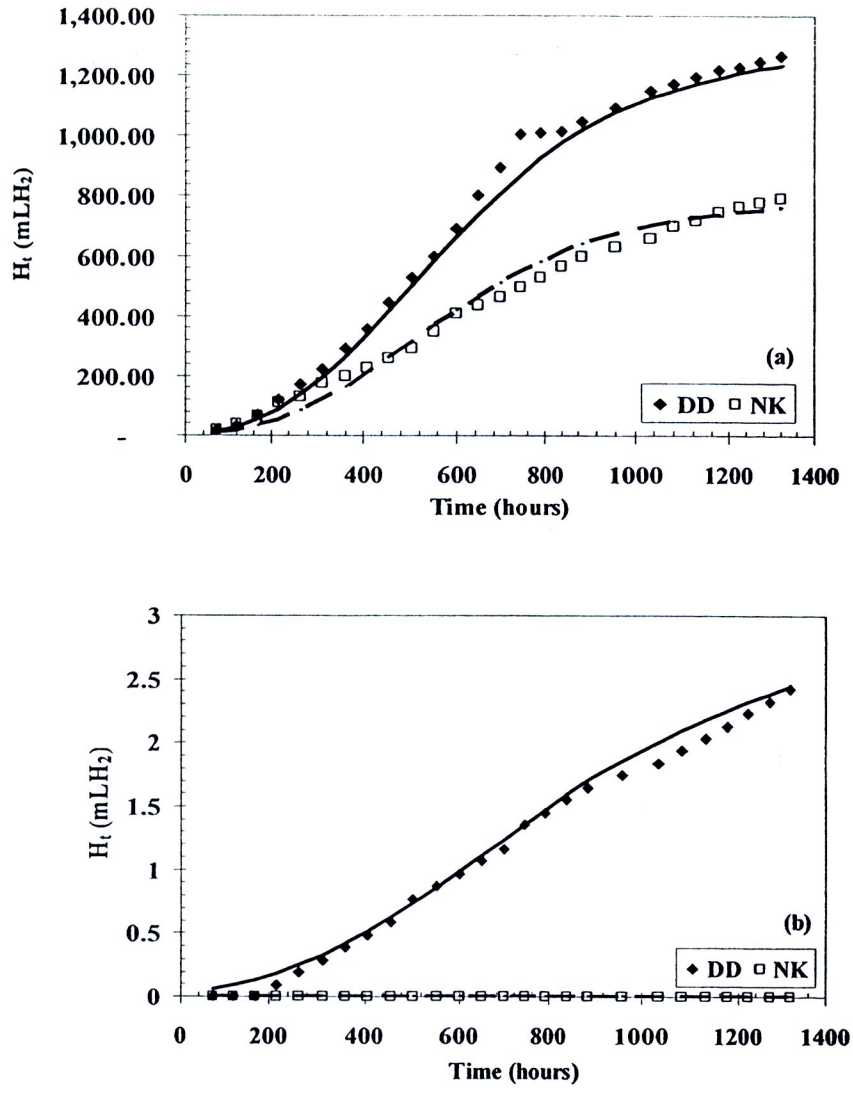

Figure 4.1 H_2 production curves during incubation in the bioreactors fed with (a) glucose and (b) starch solution

The matured inocula were employed to the batch test 1A-4A. The H_2 production curves are given in Figure 4.2. Both cultures did not produce methane (CH_4) at the pH of 5.5, confirming none of active methanogen is in the system. The treated DD sludge can effectively produce H_2, when the glucose solution was fed. The DD inocula contained a small number of active pre-fermentative microbes that can directly digest starch and produce H_2. In contrast, the treated NK sludge can produce H_2 from glucose substrate, this inoculum may be the pure culture of glucose consuming H_2 producing microbe. The biomathematical model generates the variables obtained from H_2 production curve fitting as shown in Table 4.2.

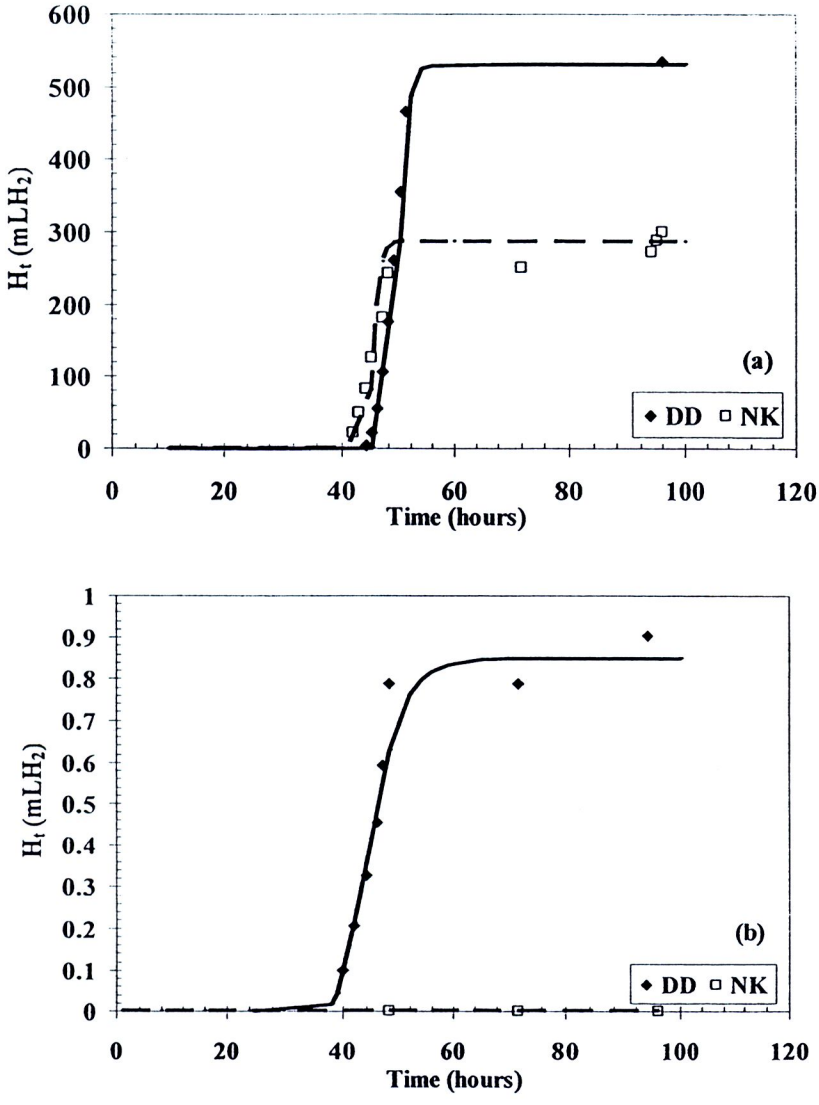

Figure 4.2 H_2 production curves in the batch bioreactors fed with (a) glucose and (b) starch solution

Table 4.2 Variables of hydrogen production process

No.	H_{max}(mL)	R (mLH_2/hour)	λ (hour)	R^2
1A	530	195	48.5	0.912
2A	287	133	44.4	0.917
3A	0.85	0.08	39.4	0.934
4A	0.00	0.00	0.00	1.000

The concentrations of glucose and the VFAs remained in the systems were examined as presented in Table 4.3. As expected, the high contents of acetate and butyrate were

obtained in reactor 1A when supplying the glucose substrate. The high amounts of glucose were obtained in reactor 3A, indicating the DD inoculum contained the group of active-fermentative microbes, which can biotransform the starch to be the simple sugar. The produced glucose in reactor 3A was further consumed by hydrogen producing microbes. Little amounts of acetate and butyrate were obtained hence the small volume of H_2 was yielded. Few amounts of glucose and acetate were observed in reactor 4A, but none of H_2 was produced. The direct hydrogen producing fermentative microbes may not involve in the NK seed. Among these inoculums, the treated DD sludge can provide the highest specific H_2 production potential at 53 $mLH_2/(gCOD/L)$.

Table 4.3 Glucose and VFAs concentrations from the bioreactor with treated sludge

No.	[Glucose] (mM)	VFAs (mM)					
		Acetate	Propionate	Valerate	Butyrate	Iso-butyrate	Total
1A	0.014	40.58	9.45	0.10	0.26	0.00	50.4
2A	0.024	26.28	1.04	0.00	0.04	0.46	27.8
3A	0.029	1.68	0.24	0.07	0.40	0.00	2.40
4A	0.022	1.11	0.04	0.07	0.00	0.00	1.21

The kinetic parameters for biohydrogen process are presented in Table 4.4. In the bioreactors 1A and 2A, the values of K_s were little higher than $C_{glucose}$ or $K_s > C_{glucose}$. This indicates the yield of H_2 did not obey the pseudo-first order kinetic reaction rate. These hydrogen producing microbes in the biosystems were operated under the substrate limitation and the specific hydrogen production rate (v_{H_2}) is relied on the concentration of glucose in the system and numbers of active microbes. On the other hand, the DD and NK biosystems fed with starch solution presented the zero order as the values of K_s were much lower than C_{starch} or $K_S << C_{starch}$. This refers the substrates did not pose any impact on yield of H_2, since the cellulytic enzyme produced by the microbes are limited or microbes are inactive to digest the long-chain cellulose molecule of starch. The uncompetitive inhibition is presented in these bioreactors.

Table 4.4 Kinetic parameters from Monod model used in treated sludge

No.	v_{max,H_2} (mLH_2/L-h)	K_S (gCOD/L)	[S] conversion efficiency (%)	COD removal efficiency (%)
1A	1.2665	21.3	30.55	83.2
2A	0.1002	18.2	20.37	78.2
3A	0.0002	0.96	0.77	76.8
4A	0.0000	0.00	0.00	74.2

The findings challenge to describe the behaviour of natural DD and NK seed, if they are fed with glucose and starch. If the hydrogen producing microbes are predominantly, they may be directly utilised as the microbial seeds.

Set II: Bioreactor 1AA-4AA was fed with mixed substrate solutions

According to the previous experiment, the maximum amount of starch utilisation was 20% of total loading. So, only 20% of starch was substituted into the mixed substrate feeding. By the same reason, the glucose substrate could be produced by the microbes at the portion of 20% of total substrate earning. So too, the 20% of glucose was replaced into the mixed substrate solution. The biosystems were fed with mixed substrate solutions. One is 80% of glucose and 20% of starch (80G+20S), the substrate concentration of 80G+20S solution is 8.2 gCOD/L. Another is80 % of starch and 20% of glucose (80S+20G), the concentration of 80S+20G is 2.8 gCOD/L.

The treated DD and NK inocula were incubated by feeding these mixed substrate solutions, until these microbes achieved the stationary phase. In case the 80G+20S synthesis wastewater was fed to the treated DD and NK inocula, they were matured within 55 days, the amounts of accumulated H_2 were 475 and 350 mLH_2, respectively. Similarly, the 80S+20G wastewater was supplied to the DD and NK inocula, they were matured within 55 days and the amounts of accumulated H_2were declined to 91 and 59 mLH_2, respectively. The decrease of cumulative H_2 might relate to the compositions and the concentration of substrate. As 80S+20G solutions has presented the lower concentration of consumable substrate than the 80G+20S solutions. Besides, the starch contains the long chain of simple sugar molecule, which is not promptly consumed by

microbes. The pre-fermentative microbes may be required to hydrolyse these complex sugar molecules to be a short chain sugar molecule, which can be directly consumed by microbial seeds. The cumulative H_2 production during incubation period is presented in Figure 4.3. The raw data of this section is shown in Appendix A.

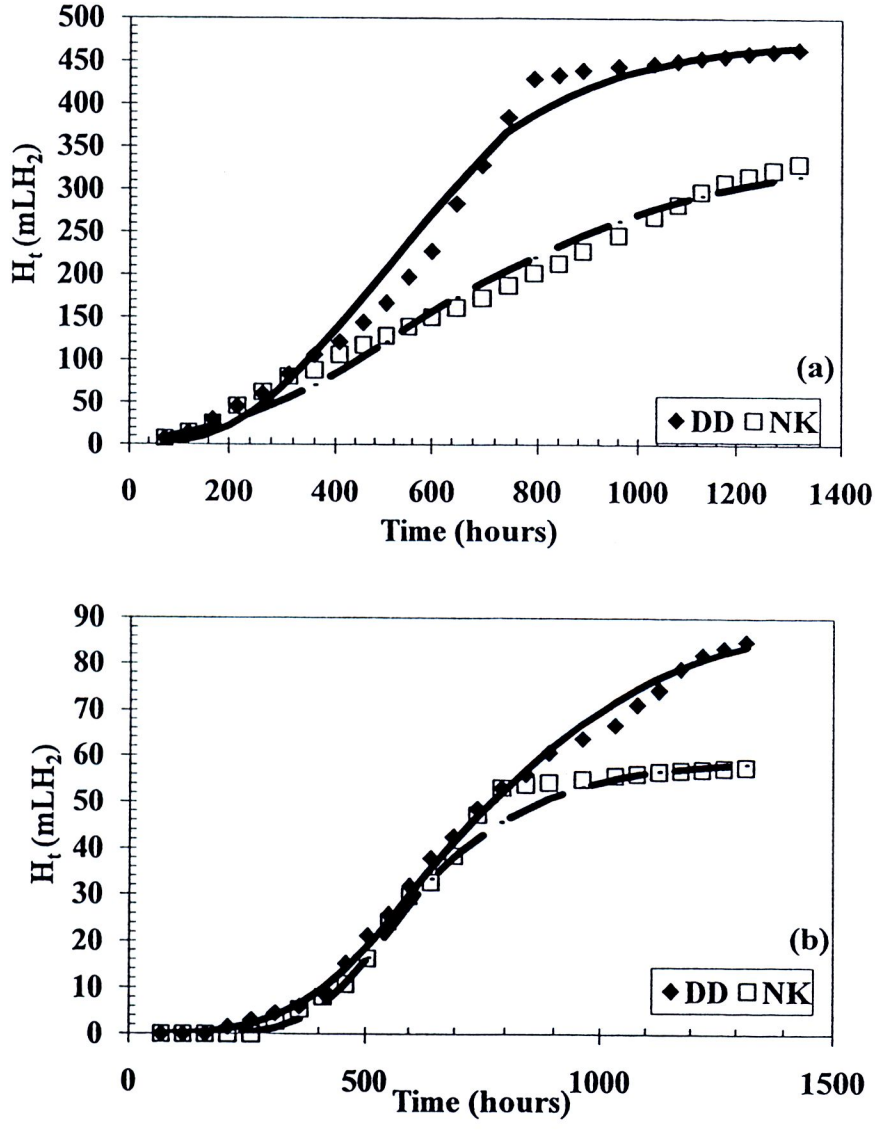

Figure 4.3 H_2 production curves during incubation in the bioreactors fed with (a) 80G+20S and (b) 80S+20G solution

The incubated DD and NK microbes were transferred to the bioreactors 1AA-4AA and the experiments were conducted similar to the previous set. The H_2 production curves are given in Figure 4.4. The biomathematical model generates the variables obtained from H_2 production curve fitting as shown in Table 4.5.

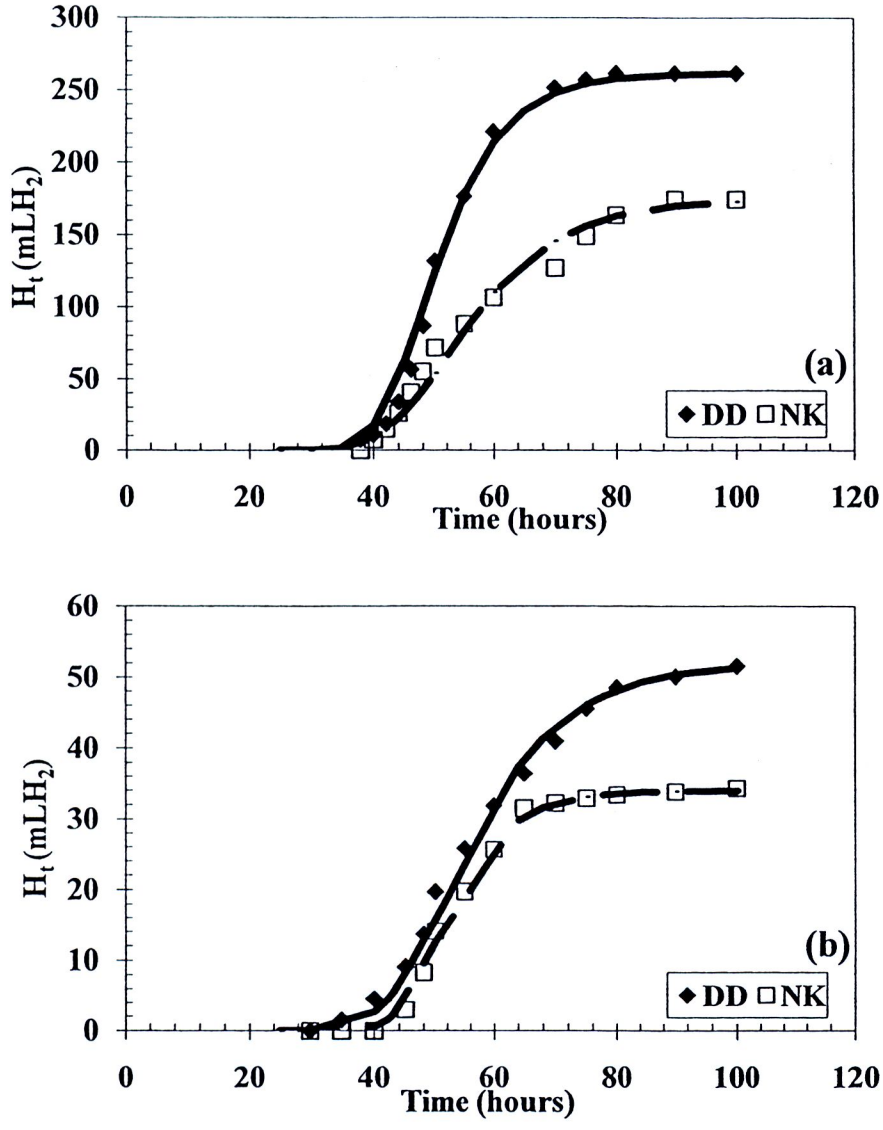

Figure 4.4 H_2 production curves in the batch bioreactors fed with (a) 80G+20S and (b) 80S+20G solution

Table 4.5 Variables of hydrogen production process

No.	H_{max}(mL)	R (mLH_2/hour)	λ (hour)	R^2
1AA	262	12.5	40	0.97
2AA	175	6.00	41	0.98
3AA	52.0	1.75	41	0.94
4AA	34.0	1.80	43	0.94

The treated DD seed can provide the highest H_2 production rate, when the 80G+20S solution was fed, as shown in bioreactor 1AA. The bioreactor 3AA, which is the DD

sludge fed with the 80S+20G solution, the small amount of H_2 was generated. By comparison, the value of H_{max} of bioreactor 1AA is only 51% of bioreactor 1A, even though, the same microbial seed was employed. The substrate concentration of 80G+20S was only 18% of pure glucose feeding stock. The value of H_{max} at bioreactor 1AA was dramatically decreased apart from the bioreactor 1A. The H_2 producing microbes could not well compete to the acidogen. This could suggest that the different substrate compositions and concentration can directly affect the H_2 production rate. Besides, the bioreactor 3AA could be enhanced by adding 20% of glucose, the H_{max} was increased 98% of bioreactor 3A. This could be confirmed the influence of substrate compositions and concentration on the microbial activity.

The treated NK microbes were employed in bioreactors 2AA and 4AA. The bioreactor 2AA had presented only 39% of H_{max} obtaining from bioreactor 2A. The bioreactor 4AA could produce the H_2 from the small amount of glucose in the mixed substrate solution. The phenomena happened in the bioreactor 4AA could suggest that the H_2 producing microbes could consume only glucose substrate. The acidogen can consume both glucose and starch. Hence, the specific substrate consumption rate was little decreased from 28.7 (bioreactor 2A) to 21.34 $mLH_2/(gCOD/L)$ (bioreactor 2AA). It can be assumed that the glucose substrate could be almost consumed by H_2 producing microbe and these microbes could slightly compete with acidogens.

The byproducts of bioreactors are volatile fatty acids (VFAs) such as acetate, propionate and butyrate. The concentrations of glucose and the VFAs remained in the systems were examined as presented in Table 4.6.

Table 4.6 Glucose and VFAs concentrations from the bioreactor with treated sludge

No.	[Glucose] (mM)	VFAs (mM)					
		Acetate	Propionate	Valerate	Butyrate	Iso-butyrate	Total
1AA	0.014	20.7	13.3	0.18	0.28	0.00	34.36
2AA	0.023	11.3	1.27	0.86	0.00	0.00	13.45
3AA	0.031	4.77	1.32	0.00	2.65	2.69	11.43
4AA	0.019	9.79	0.49	0.33	0.00	0.00	10.61

The amount of glucose produced in bioreactors 1AA-4AA was same as the previous set of bioreactors 1A-4A. The total VFAs produced in the bioreactors 1AA was the highest concentration and the acetate and propionate were predominant byproducts. Similarly, the bioreactors 2AA had predominantly produced acetate and propionate. Although the different inoculum was employed, the same VFAs compositions were obtained. This could reflect the influence of substrate compositions on the metabolic pathway of microbes, resulting in the same VFAs compositions. Apart from this finding, the VFAs byproduct concentrations were inversely ordered with the numbers of carbon atoms. This suggested that the catabolism may play an important role in metabolism process of these inocula. However, the microbial seeds of these bioreactors could generate the lower amount of VFAs than the bioreactors 1A and 2A.

The bioreactors 3AA and 4AA had shown the higher amount of VFAs byproducts than the bioreactor 3A and 4A. The compositions of VFAs in bioreactor 3AA and 3A were same, the acetate and butyrate were predominantly yielded. The bioreactor 4AA and 4A had mainly produced the acetate and propionate. So, the glucose could enhance the activity of H_2 producing microbe and the same metabolic pathways were still occurred in these bioreactors. The higher acetate and butyrate byproducts of bioreactor 3AA had referred to the higher H_2 production rate. The acetate and butyrate concentrations were increased from 2.08 mM in bioreactor 3A to 10.11 mM in bioreactors 3AA, or increasing 79.4% of the case of pure starch feeding. However, the microbes in bioreactor 3AA could increase the H_2 yield 98% higher than the bioreactor 3A. It could be implied that the H_2 producing microbe may earn substrate from glucose and starch. On the other hand, the bioreactor 4AA could produce acetate at 9.79 mM, the expected H_2 yield was 39.16 mM or 877 mL H_2, but the obtained H_2 yield was only 34 mL. The obtained H_2 yield was much lower than the expected, since the treated NK inoculum may contain low amount of H_2 producing bacteria, but this seed may be predominantly comprised of acidogen.

Although the H_2 gas is obtained as the product of these bioreactors 1AA-4AA, the source of substrate could not be directly declared. Neither glucose nor starch concentration could not be used in term of substrate concentration in the Monod model. The COD was presented the whole concentration of substrate in the Monod model. The kinetic parameters from Monod model used in treated sludge are summarised as presented in Table 4.7.

Table 4.7 Kinetic parameters from Monod model used in treated sludge

No.	v_{max,H_2} (mLH_2/L-h)	K_S (gCOD/L)	[S] conversion efficiency (%)	COD removal efficiency (%)
1AA	0.0029	3.95	13.98	81.42
2AA	0.0397	3.57	11.05	73.39
3AA	0.0044	1.87	8.33	73.21
4AA	0.0001	1.22	5.69	72.79

The values of v_{max,H_2} of bioreactors 1AA and 2AA were much lower than the ones obtained from bioreactors 1AA and 2AA. The lower values of v_{max,H_2} could represent the noncompetitive product inhibition, which starch can inhibit the hydrogenase enzyme. Above this finding the bioreactors 1AA and 2AA had presented the growth limitation dealing with the limit numbers of H_2 producing microbes, as the $K_s < C_{substrate}$ was presented in both bioreactors. Besides, the values of v_{max,H_2} obtained from bioreactors 1AA and 2AA are very far away from those ones given by the bioreactors 1A and 2A. This exhibited that these bioreactors suffered from the noncompetitive product inhibition, which reflected that the activity of hydrolase enzyme may be blocked with the starch substrate. The substrate conversion efficiencies of bioreactors 3AA and 4AA were lower than the theoretical values. This may be the influence of substrate compositions and concentrations, as these factors could depress the activity of H_2 producing microbes. This could be supported the statements, which were made by the Gompertz model and VFAs byproducts analysis.

In the inverse directions, the bioreactors 3AA and 4AA had the higher values of v_{max,H_2} than the bioreactors 3A and 4A. The inhibition from substrate limitation could not harm the microbes. However, the value of v_{max,H_2} of bioreactors 3AA was higher than one from the bioreactor 1AA. This could refer the influence of substrate concentration on the growth of H_2 producing bacteria. Only small concentration of glucose was decreased in bioreactor 1AA, the activity of H_2 producing bacteria had been significantly disturbed. Inversely, adding some small amount of glucose in the mixed solution, the

H_2producing microbe could be significantly enhanced as shown in bioreactor 3AA. The value of v_{max,H_2} of bioreactor 2AA was higher than the one presented by bioreactor 4AA, although the treated NK seed was employed in both bioreators. This could be implied that the H_2 producing microbes containing in NK seed preferred to utilise glucose rather than starch. So, the activity of H_2 producing microbe in bioreactor 4AA could not be boosted up by adding glucose. Moreover, the values of K_s at bioreactors 3AA and 4AA were slightly lower than the substrate concentration, indicating that the growth of H_2 producing microbe could directly influence the yield of H_2. If the numbers of H_2 producing microbe was multiply enlarged, the H_2 yield could be increased. However, this condition could be performed only in bioreactor 3AA, since the high substrate conversion efficiency.

4.2.2 Natural inoculums

Set III: Bioreactor 1B-4B was fed with pure glucose and pure starch solutions

The matured inocula of DD and NK were employed to the batch test 1B-4B. Even pure glucose and starch solutions were fed the biosystems, these bioreactors did not generate the H_2. The modified Gompertz equations were applied to estimate the concentration of acetate and butyrate instead of H_2 gas to define the relationship between VFAs producing rate over time. As the major VFAs of these biosystems were acetate and butyrate, which reflected the mechanisms of both H_2 producing microbe and acidogens. The cumulative of acetate and butyrate curves are given in Figure 4.5. The natural DD sludge could effectively produce acetate and butyrate, when the glucose solution was fed. The DD inoculum contained a small number of active acidogenesis microbes that could directly digest starch and produce VFA. In contrast, the treated NK sludge could produce acetate and butyrate from glucose substrate, this inoculum may be the pure culture of glucose consuming acidogenesis microbe. The VFAs producing rate (R_p) reflected that the microbes prefer to uptake glucose rather than starch. The biomathematical model generates the variables obtained from acetate and butyrate curve fitting as shown in Table 4.8.

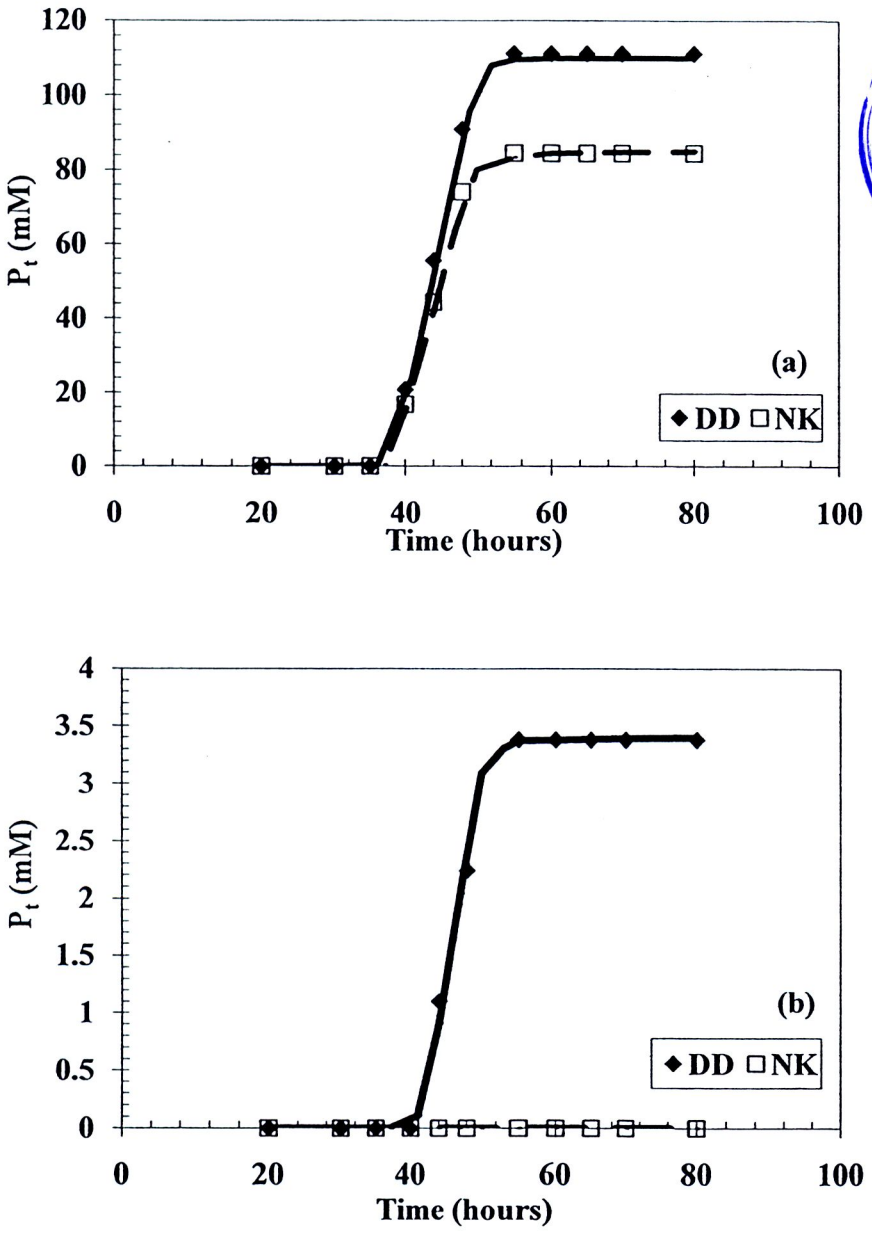

Figure 4.5 VFAs producing curves in the batch bioreactors fed with (a) glucose and (b) starch solution

Table 4.8 VFAs concentrations yielded from the bioreactor with natural sludge

No.	P_{max}(mM)	R_p (mM/hour)	λ (hour)	R^2
1B	110	12.0	38.0	0.991
2B	85.0	7.00	38.0	0.993
3B	3.40	0.30	41.0	0.934
4B	0.00	0.00	0.00	1.000

Both the natural inoculums of DD and NK produced a very low amount of H_2 production every bioreactors during the incubation. Apart from the incubation periods, the behaviour of the natural DD and NK inocula were observed via a batch test. The natural DD and NK inocula did not produce any H_2 and CH_4 gas, even the observation period was very long. This could confirm that the hydrogen produce microbe could not compete with the other microbes. Moreover, the methanogen was inactive in this control acidic condition (pH=5.5). The natural DD and NK inocula could produce glucose and VFAs to the biosystem. The concentrations of glucose and VFAs in the system are presented in Table 4.9.

Table 4.9 Glucose and VFAs concentrations from the bioreactor with natural sludge

No.	[Glucose] (mM)	VFAs (mM)					
		Acetate	Propionate	Valerate	Butyrate	Iso-butyrate	Total
1B	0.014	34.47	8.93	0.00	0.32	0.00	43.72
2B	0.210	18.69	1.97	0.00	3.70	0.46	24.82
3B	0.024	9.08	0.10	0.00	0.00	0.00	9.18
4B	0.012	0.00	2.63	0.00	0.00	0.00	2.63

The VFAs were highly yielded, when the biosystems were fed with glucose. The Gompertz model could suggest the same agreement as the observation. The natural DD seed could highly biotransform the glucose substrate to be VFAs comparing to the natural NK seed. Similarly to the biosystem with glucose feeding, the natural DD seed could present the higher efficiency of VFAs producing than the natural NK seed. Besides, the natural DD inoculum had some numbers of cellulosic enzyme producing microbe. The long chain of starch was turned to be the glucose and some produced glucose could be further consumed by the acidongens, generating the VFAs into the biosystem.

The sCOD removal and [S] conversion efficiency (%) of the natural DD and NK microbial seed are summarised as presented in Table 4.10. The natural DD and NK inocula could provide the high substrate conversion and sCOD removal, when the glucose solution was fed. This can confirm that the natural DD and NK inocula may

contain the active fermentative microbes that could effectively digest the simple sugar substrate. Only the natural DD may consist of cellulytic enzyme producing microbe, which can contribute the glucose to the biosystem. The glucose may be obtained from the biotransformation of starch substrate. However, this glucose was consumed by acidogens, and then the VFAs were yielded.

Table 4.10 Substrate conversion and sCOD removal in natural inoculums

No.	[S] conversion efficiency (%)	sCOD removal efficiency (%)
1B	0.29	71.2
2B	0.19	68.5
3B	0.00	70.0
4B	0.00	69.5

The findings can be implied that the treated DD seed was consisted of the glucose consuming H_2 producing microbe and some of the active microbes could directly biotransform the long chain of starch to be glucose and VFAs. The natural DD may contain the pre fermentative microbes that could consume both of glucose and starch. However, the activity of pre-fermentative microbes could give the low yield of glucose. Besides, glucose generated from the cellulosic digesting microbe was tended to be converted to VFAs by the acidogens, which did not give the H_2 gas. In order to use the DD inoculum in the biohydrogen reactor, the heat treatment was recommended. The heat treatment can eliminate the unwanted species, such as the acidogens and methanogens. However, the substrate selective may be useful to enhance the growth of specified species in the proper pathway. The heat shock coupled with substrate selection may be employed to classify the H_2 producing microbes from the mixed culture.

Set IV: Bioreactor 1BB-4BB was fed with mixed substrate solutions

The mixed substrate solutions were employed to bioreactors 1BB-4BB with the same function as the bioreactors 1AA and 4AA, but the untreated inocula were employed. These bioreactors had produced the low amount of H_2, hence the concentrations of acetate and butyrate were represented the VFAs production rate, instead of H_2 production rate. The cumulative concentration of VFAs over time of each bioreactor is given in Figure 4.6. The constants of Gompertz model are provided in Table 4.11.

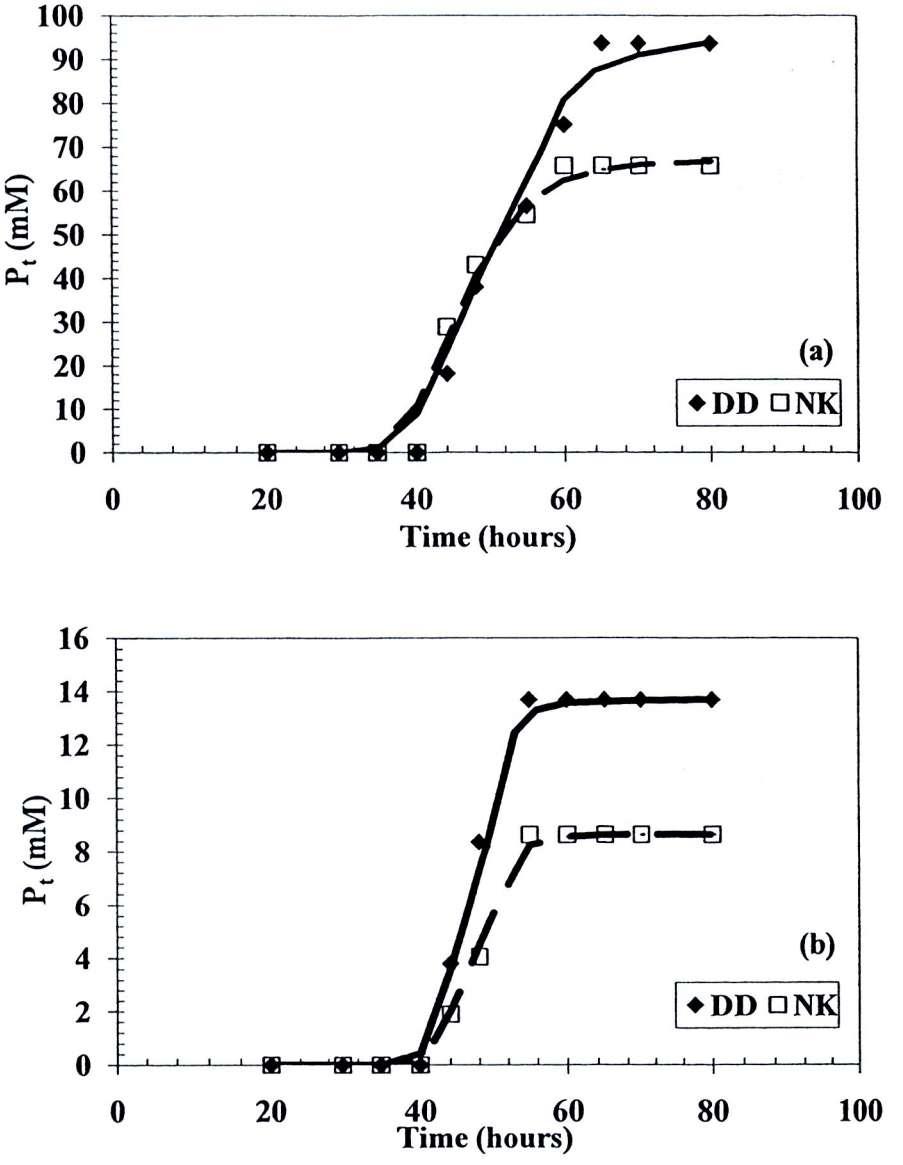

Figure 4.6 VFAs producing curves in the batch bioreactors fed with (a) 80G+20Sand (b) 80S+20G solution

Table 4.11 VFAs concentrations yielded from the bioreactor with natural sludge

No.	P_{max}(mM)	R_p (mM/hour)	λ (hour)	R^2
1BB	95.0	4.70	39.00	0.984
2BB	67.0	4.00	37.50	0.985
3BB	13.7	1.20	41.00	0.997
4BB	8.65	1.00	42.00	0.984

The values of P_{max} of bioreactors 1BB and 2BB were slightly lower than the ones from bioreactors 1B and 2B. Even though the substrate concentration of the mixed substrate solution was reduced 18% of the pure glucose solution, the values of P_{max} of bioreactors 1BB and 2BB were reduced 13 and 21% from the bioreactors 1B and 2B, respectively. Therefore, the substrate concentration and compositions could less impact the bioactivity of untreated microbial seed. Since, these microbes could consume both glucose and starch substrates, the values of R_p of bioreactors 1BB and 2BB were insignificantly different, this could refer the rates of substrate consumption of these bioreactors were same.

The bioreactors 3BB and 4BB had presented the different behaviour, they could produce higher amount of VFAs than the bioreactors 3B and 4B. The concentration of the mixed substrate solution was 64% higher than the pure starch solution, but the VFAs production rates were increased 75% and 100% of the rates obtained from bioreactor 3B and 4B, respectively. The glucose could slightly increase the activity of acidogen. Otherwise, the H_2 producing microbe could not compete with acidogen. The values of R_p were increased in bioreactor 3BB and 4BB, when adding more glucose in the mixed substrate solution. By comparison, the values of P_{max} and R_p of bioreactors 3BB and 4BB were less than the ones given by bioreactors 1AA and 2AA. This indicated that the suitable way to enhance the activity of acidogen was increasing the concentrations of substrate.

The H_2 yields of bioreactors 1BB and 2BB were slightly lower than the bioreactors 1B and 2B. On the other hand, the H_2 yield at bioreactors 3BB and 4BB were increased from the bioreactors 3B and 4B. Therefore, the compositions of VFAs and the concentration of obtained glucose in these biosystems were the key parameter to describe this finding. The observation data are given in Table 4.12.As expected the high concentrations of glucose were presented in bioreactors 1B and 2B rather than the bioreactors 1BB and 2BB. In addition to the concentration of glucose at bioreactors 3BB and 4BB were higher than the reactor 3B and 4B. This informed that the starch could be partly converted to be glucose, when the biosystem had been supplied with some amount of glucose. The microbes tended to use glucose in the feedstock rather than the produced glucose via the pre-fermentation. The biosystem 3BB with high

concentration of starch feedstock, the pre-fermentative could produce some amount of glucose to maintain the substrate level in the bioreactor. Unlikely, the bioreactor 4BB was slightly produced glucose, even though the microbes were served with small amount of glucose. The pre-fermentative microbe in the untreated DD seed may be more highly active than the untreated NK. The concentrations of acetate and acetate at the bioreactors 1AA and 2AA were 18.9 and 18.5 mM, which were insignificant different. However the values of P_{max} of these bioreactors 1AA and 2AA were totally different. This could be claimed that the microbes in the bioreactor 2AA may present the different pathway, which could return propionate into the bioreactor. However, these acidogens did not generate H_2 or CH_4 gas. Similarly, the biorectors 3BB and 4BB could provide the same concentration of VFAs, but different compositions. The bioreactor 4BB could mainly produce the propionate, but the bioreactor 3BB could generate mainly acetate as the byproduct. This could reflect the activity of H_2 producing microbe in the untreated biomass. The natural DD sludge had contained few numbers of H_2 producing microbes, but they were mainly contained acidogen that could stimulated the hydrogen producing microbe by producing glucose via the pre-fermentation.

Table 4.12 Glucose and VFAs concentrations from the bioreactor with natural sludge

No.	[Glucose] (mM)	VFAs (mM)					
		Acetate	Propionate	Valerate	Butyrate	Iso-butyrate	Total
1BB	0.019	18.7	9.82	0	0.16	0	28.69
2BB	0.021	14.1	3.01	0	4.09	0.26	21.49
3BB	0.022	4.18	1.01	0	0.39	0	5.58
4BB	0.014	2.76	2.51	0	0.19	0	5.46

By comparison, the compositions of VFAs in the bioreactors 1BB-3BB were similar to the bioreactors 1B- 3B. Only, the bioreactors 4BB had presented the compositions differently from the bioreactor 4B. The activities of microbes bioreactor 1BB-3BB may be conserved in the same pathway. The high concentration of glucose did only enhance the VFAs production rate of untreated DD and NK inocula. Besides, the untreated DD inoculum was considered as the uniform microbe species, so the pathway of microbe

was preserved although the different compositions of substrates were served. The untreated NK inoculum did not obey the same pathway as they were the mixed cultures and there was no predominant species.

The Monod model was served the calculation of kinetic parameters for both of pre-fermentation and biohydrogen process. The calculated results are summarised in Table 4.13.

Table 4.13 Kinetic parameters from Monod model used in natural sludge

No.	v_{max,H_2} (mLH_2/L-h)	K_S (gCOD/L)	[S] conversion efficiency (%)	COD removal efficiency (%)
1BB	0.0002	3.05	0.41	70.27
2BB	0.0002	4.33	0.33	69.38
3BB	0.0001	1.32	0.61	70.55
4BB	0.0005	10.6	0.57	68.35

All bioreactors in this set had present the same figures of v_{max,H_2}. The values of v_{max,H_2} were lower than the Sets I and II. The untreated DD and NK inocula were mixed cultures, so the H_2 microbe was less active and their numbers was lower than the treated inocula. The values of K_s of bioreactors 1BB-3BB had presented that the activities of H_2 producing microbe and acidogens were controlled by the concentrations of substrates, but the bioreactor 4BB did not obey this rule. The activities of untreated NK seed were controlled by both of substrate concentrations and numbers of microbes. The glucose and starch substrates could be consumed by both H_2 producing and pre-fermentative microbes and they generated the VFAs byproduct only.

Based upon these findings, the treated DD and natural DD seeds seemed to be suited for biohydrogen and pre-fermentative, respectively. The treated DD seed had contained H_2 producing microbes, which could consume both of glucose and starch, as well as this seed had some numbers of acidogen. The natural DD seed had contained the pre-fermentative microbes predominantly, this inoculum could produce glucose, acetate and

butyrate as product and byproduct, when these cells were served with starch. Hence, these natural and treated sludges were further employed to the Series 2.

4.3 Series 2: Anaerobic Sequencing Batch Reactor

According to the previous experiment, the active and acclimatised hydrogen producing micobes and acidogens were selected. As considered earlier, the treated DD and natural DD inocula did not present the same favourable substrate condition. The expected pathways for biodegradation of starch solution, the starch should be firstly converted to glucose and VFAs via the pre-fermentation process. Then, the produced glucose and the remaining starch substrates should be consumed by H_2 producing microbe via the dark fermentation. The feeding solution for the dark fermentation composted of mixed substrates of glucose and starch, so this substrate solution could either enhance or retard the activities of H_2 producing microbe as discussed earlier. The natural DD was able to consume the starch as substrate, unless the acidogenesis microbes could not earn the glucose. The H_2 producing microbe of these natural inocula may little compete with the acidogen, under the presence of glucose substrates in the pre-fermentation stage. Additionally, the H_2 producing microbes in the treated DD seed prefer to the enrichment of glucose substrate. The H_2 producing microbes could be enhanced in the second stage of dark fermentation. These all supporting reasons brought the design of the two stages bioreactor, which were pre-fermentation and dark fermentation. The two pairs of bioreactors were operated as the anaerobic sequencing batch reactor (ASBR).

In the first pair, the symbols of DD1 and DD2 referred to pre-fermentative and dark fermentative bioreactor, respectively. Likely, the second pair the DD3 and DD4 were pre-fermentative and dark fermentative bioreactor, respectively. Both pairs were fed with starch solution at a concentration of 1gCOD/L. After 66 days incubating, theinocula of these bioreactors were enough matured. The amounts of accumulated H_2 were 4727 and 4064 mLH_2 for the bioreactors DD1 and DD3, respectively. The accumulated H_2were 697 and 595 mLH_2 for the bioreactors DD2 and DD4, respectively. The cumulative H_2 production during incubation period of the pre-fermentation and dark fermentation of pairs 1 and 2 are presented in Figures 4.7 and 4.8, respectively. The raw data is shown in Appendix B. The results had been presented differently from the expectation as the pre-fermentation process could contribute the higher cumulative

H_2 than the dark fermentation. This finding did not correlate with the results obtained from the Series 1. The reason for this claim may associate with the bioactivities of acidogens and H_2 producing microbes, since the plenty amounts of starch were fed to the active acidogen, returning glucose as substrate in the first stage at pre-fermentor. The obtained glucose could be directly consumed by the H_2 producing microbes. In the meantime, some portions of starch could be degraded by H_2 producing microbes. Under the plenty substrates, the natural DD inoculum could be enhanced or highly activated. The effluent discharged from the pre-fermentation may contain low amounts of glucose and starch substrates. The dark fermentative microbes in bioreactor DD2 and DD4 may not gain enough substrate. These microbe may starve, therefore the yield of H_2 was dramatically reduced. Even the same inoculum was employed to these pairs of bioreactors, the different H_2 yield was presented. The yields of H_2 of bioreactors DD1 and DD3 were resemblance, but the figures of H_2 yield at bioreactors DD2 and DD4 were totally different. This could strongly confirm that the concentration of substrates could affect straightly to activities of H_2 producing microbes in the dark fermentation process rather than the pre-fermentative microbes. Otherwise, the active acidogen in pre-fermentor could tolerate the changes of substrate concentrations and compositions. As long as the acidogen could resist these changes, the H_2producing microbes in the pre-fermentation could earn the substrates. By observation, the cumulative volumes of $H_2$of the pre-fermentation stage did not reach the maximum values as the exit point of s-curve had not been presented. This could point that the activities of H_2 producing microbes at the pre-fermentator were dynamically droved by the concentrations and compositions of substrates generated by the species of acidogen. Inversely, the H_2 producing microbes in the second stage at the dark fermentor were highly sensitive to the changes of concentration and compositions of substrates. The completed s-curve of H_2 production curve did not observe, same assumption was made dealing with the impact of substrate supplying. The activities of H_2 producing microbes at the dark fermentation stage had undergone with the reductions of substrate concentration, especially glucose. Since the amount of glucose was higher than the minimum concentration for consumption, the H_2 producing microbes could firstly utilise glucose as the primary substrate. If the glucose concentration went under the consumable level,H_2 producing microbes may utilise starch as the secondary substrate. These findings had indicated that the substrate distribution in the mixed microbial species are significant factors with respect to the metabolic pathways and bringing the different

constituents of the products and byproducts. In this case, the digested starch may exist in mixed forms of glucose and VFAs that could be appropriately stimulate the target substrates in the different forms within consumable through the dark fermentation process. Thus, the spatial and temporal profiles of bioreactors DD1-DD4 separately from those conceptual representation of Series I.

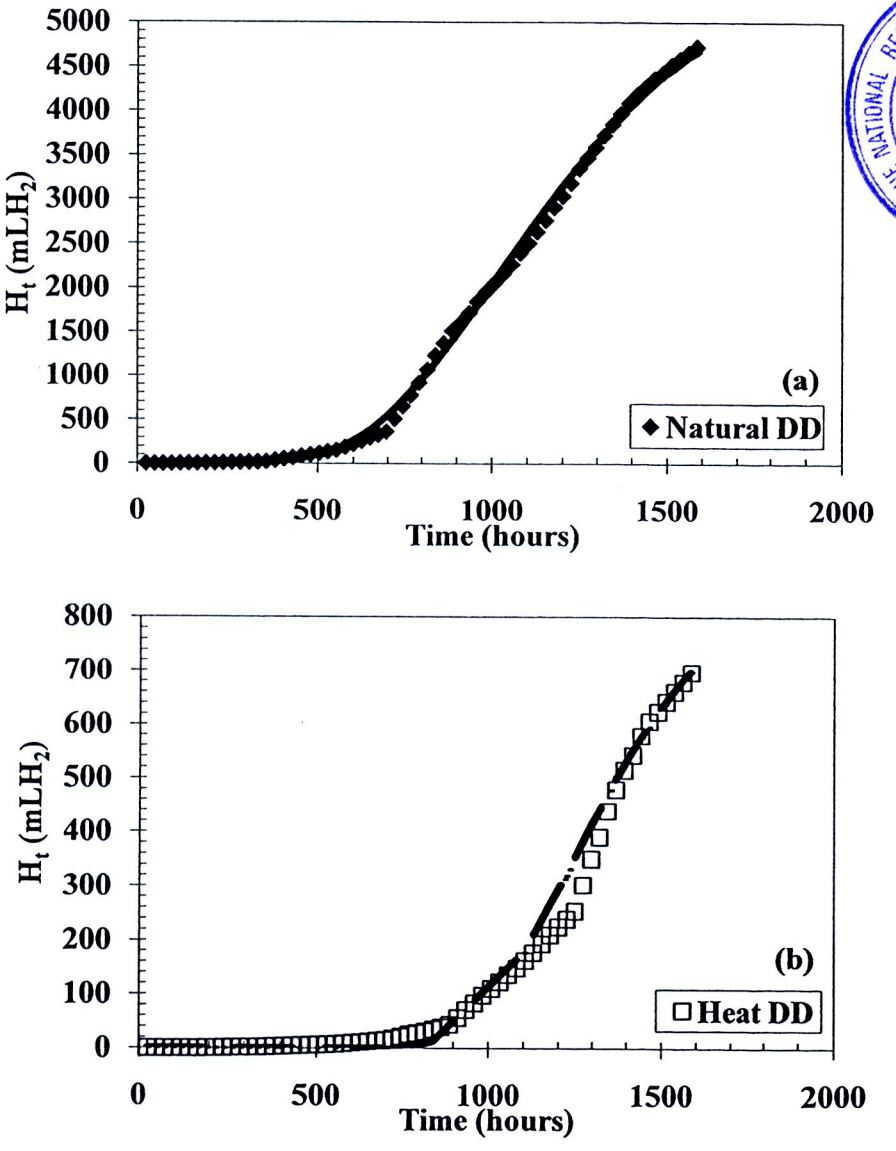

Figure 4.7 H_2 production curves in the two stages biohydrogen (a) pre-fermentative DD1 and (b) dark fermentative DD2

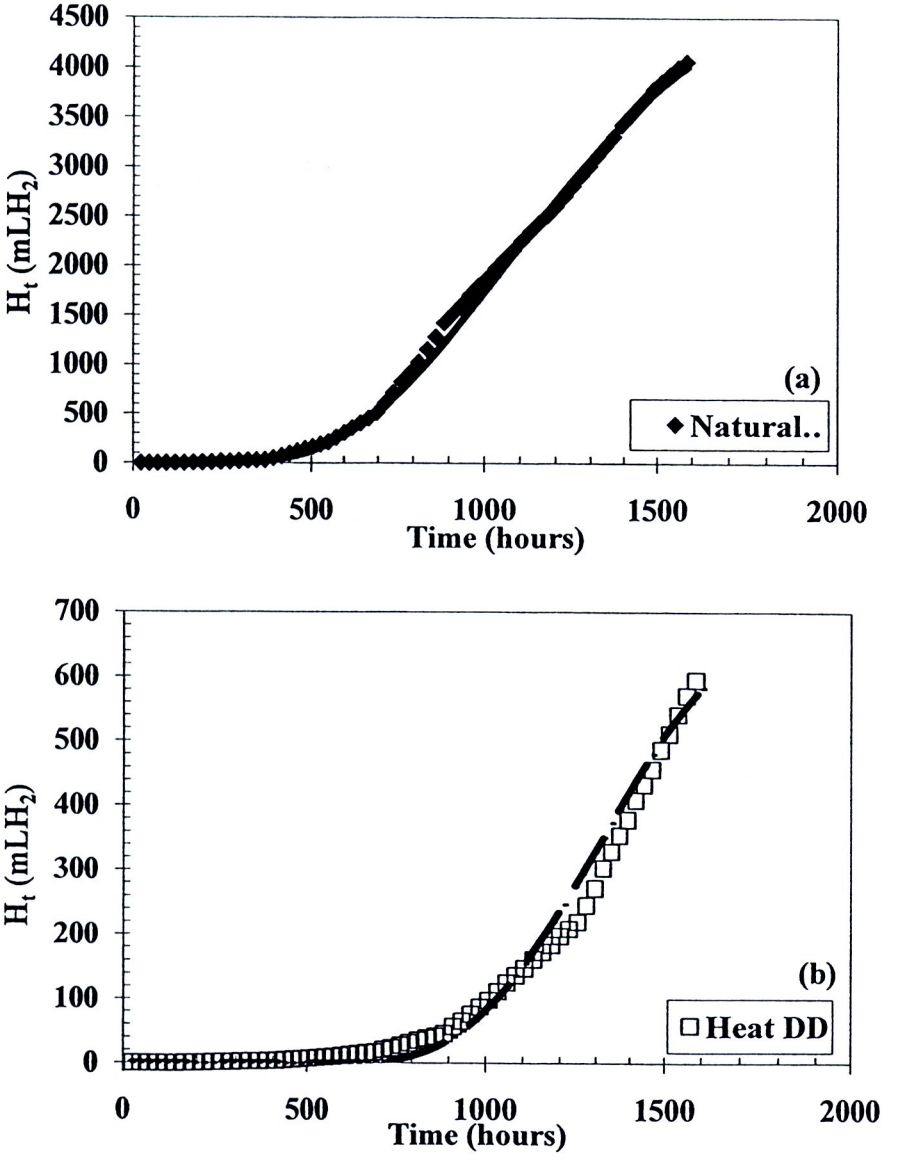

Figure 4.8 H_2 production curves in the two stages biohydrogen(a) pre-fermentative DD3 and (b) dark fermentative DD4

The biological H_2 performance of matured inocula from bioreactors DD1-DD4 were examined via the modified Gompertz model. The results are given in Figure 4.9. These cultures did not produce methane (CH_4), the same conclusion could be drawn that none of active methanogen was in these biosystem. The starch consuming H_2 producing microbes containing in the natural DD inoculum could be stimulated under the substrate enrichment. The active acidogens also contribute the highly concentrated glucose substrates to the biosystems. The glucose consuming H_2 producing microbes could be boosted up by the produced glucose by the pre-fermentative microbes. These species of H_2 producing microbes could contribute a high yield of H_2, since the first stage. The

treated DD inocula that predominantly contained the H_2 producing microbes, these active microbes may undergo the substrate limitation or the low ratio of food to microorganism (F/M), delivering the low H_2 production rate.

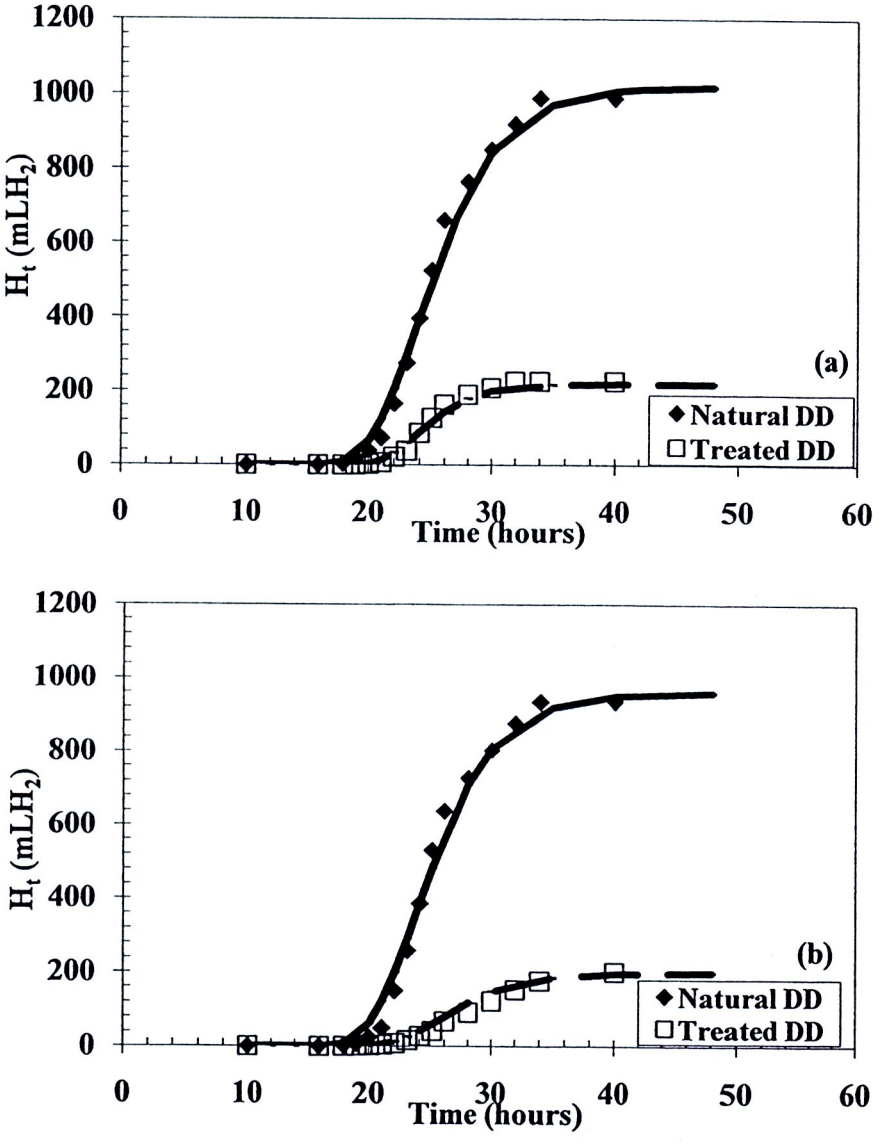

Figure 4.9 H_2 production curves in the two stages biohydrogen process
(a) DD1 and DD2 (b) DD3 and DD4 bioreactors

The biomathematical model had generated the constants of bioactivities in the H_2 production processes as shown in Table 4.14.

Table 4.14 Variables of hydrogen production process in the two stages biohydrogen

No.	H_{max}(mL)	R (mLH_2/hour)	λ (hour)	R^2
DD1	1020	100	20	0.983
DD2	220	30	21	0.990
DD3	960	97	20	0.977
DD4	200	20	22	0.984

The first pair of bioreactors had presented the higher figures of H_{max} and R than the second pair of bioreactors. These variables could imply that the first pair of bioreactors DD1 and DD2 may contain the more numbers of active species of acidogens and H_2 producing microbes than the second pair of bioreactors DD3 and DD4. To be ensured with this statement, the concentrations of glucose and the VFAs remaining in the systems were examined as presented in Table 4.15. As expected, the high contents of acetate and butyrate were observed in the pre-fermentative stage at bioreactors DD1and DD3. The high amounts of glucose were also observed in the per-fermentation process at the bioreactors DD1 and DD3. This could strongly support that the natural DD seeds were the actively fermentative microbes, which could biotransform the starch to be the simple sugar. The produced glucose in bioreactors DD1 and DD3 was further consumed by hydrogen producing microbes at the bioreactors DD2 and DD4. The bioreactors DD2 and DD4 had returned some amounts of acetate and butyrate as byproducts. However, the pre-fermentative and dark fermentative microbes of the pair of bioreactors DD1 and DD2 seemed to be the more highly activated than another pair of bioreactors. This finding was well confirmed the statement made via the modified Gompertz model. With starch feedstock, the two stage biohydrogen could contribute the H_2 at 1240 and 1160 mLH_2/(gCOD/L) in the first and the second pair, respectively. The rate of H_2 production in the pre-fermentation was 5 times more quickly than the dark fermentation. The way to enhance the dark fermentation process may be done by adding some substrates to level up the F/M ratio.

Table 4.15 Glucose and VFAs concentrations from two stages biohydrogen

No.	[Glucose] (mM)	VFAs (mM)					
		Acetate	Propionate	Valerate	Butyrate	Iso-butyrate	Total
DD1	0.061	25.37	1.95	1.13	0.32	2.87	31.64
DD2	0.041	24.51	0.80	0.77	0.33	3.22	29.62
DD3	0.061	23.47	2.03	0.78	0.27	1.87	28.41
DD4	0.045	23.17	2.11	0.73	0.22	2.50	28.73

The overall efficiency of the two stages biohydrogen are summarised in the Table 4.16.The specific H_2 yield was calculated based on the theoretical molar ratio of H_2 to glucose. The H_2 yields of the two stage biohydrogen were 0.28 and 0.32 mole H_2/mole glucose for the first and the second pair bioreactor, respectively. These obtained H_2 yield were far away from the theoretical yield. This may the result of the unattractive substrate, which was starch. By comparison to the previous research, these batch scale bioreactors could satisfactorily contribute the yield of H_2.

Table 4.16 Performance of two stages biohydrogen

No.	H_2 yield (mol H_2/mol glucose)	[S] conversion efficiency (%)	% sCOD Removal	Final VSS (g/L)
DD1	0.261	6.52	68.12	7.600
DD2	0.123	3.06	60.98	5.667
DD3	0.222	5.55	65.22	7.233
DD4	0.109	2.72	56.52	5.433

The Monod model was served the calculation of kinetic parameters for both of pre-fermentation and dark fermentation at the two stages biohydrogen process. The calculated results are summarised in Table 4.17.Every figures had been represented the same characteristics observed via the modified Gompertz model. The values of v_{max,H_2} at the pre-fermentors DD1 and DD3 were much higher than the dark fermentors DD2 and DD4. In the latter assumption, the critical point of concern was the reaction and the release of substrate species from individual DD1 and DD3 bioreactors could be

distinguished the beginning of substrates fed through the dark fermentors DD2 and DD4, respectively. These two pairs biosystems was simultaneously conducted from the start of the pre-fermentation until steady state of dark fermentation was reached, the magnitudes of v_{max,H_2} in pre-fermentative stage were remained more highly than the dark fermentative stage. The yield of H_2 at the bioreactors DD1 and DD3 were relied on the substrate concentration and numbers of active acidogen and H_2 producing microbes since the $K_S > C_{starch}$. The yield of H_2 of bioreactors DD2 and DD4 had corresponded with the number of active H_2 producing microbes, as $K_s << C_{starch}$. By comparison, the values of v_{max,H_2} obtained from the pre-fermentator DD1 and DD3 were greater than bioreactor 3B, which was 0.00 mLH_2/L-h. This pointed out that the inocula in pre-fermentor DD1 and DD3 could digest starch without substrate limitation. This approach described herein was relevancy the prolonged microbes may completely adapt themselves to be familiar with starch. The metabolic pathway was well functioned to meet the target, consuming starch via H_2 respiration. The obtained v_{max,H_2} values at dark fermentors DD2 and DD4 were higher than both bioreactor 1BB and 3BB. The inocular in the dark fermentation overwhelmed the substrate inhibition, since the long term aging the more effective H_2 producing microbes were survive.

Table 4.17 Kinetic parameters from Monod model from two stages biohydrogen

No.	v_{max,H_2} (mLH_2/L-h)	K_S (gCOD/L)
DD1	0.0052	1.10
DD2	0.0007	0.32
DD3	0.0058	1.08
DD4	0.0008	0.28

4.4 Summary

The heat shock treatment was applied to reduce the unwanted species in DD and NK seeds and followed by the substrate selective techniques. The natural DD inoculum had contained the highly active acidogen, which could digest the starch and return glucose and VFAs as the product and byproduct, respectively. However, the untreated NK seed

contained less active acidogenic species, the rate of starch conversion was relatively low. The treated DD sludge had the mixed species of H_2 producing microbes, which could consume both of glucose and starch as substrate and they could contribute a high H_2 yield via the dark fermentation. On the other hand, the treated NK inoculum could be specific with glucose substrate. The treated NK seed was relatively inactive, the low H_2 yielded was presented at the dark fermentation.

The two stages biohydrogen reactors was designed, the pre-fermentor was fed with starch solution. The effluent of pre-fermentor was passed through the dark fermentor as the feedstock. The natural and treated DD seeds were employed to the pre-fermentative and dark fermentative stage, respectively. The expectation was made in associated with the previous test of culture selection. The pre-fermentative microbe might digest starch and then they could distribute the glucose and other short chain sugars to the biosystem. The H_2 producing microbes in the second stage at the dark fermentor could earn the glucose and other short chain sugars from the wastewater discharged from the pre-fermentor. The maximum yield of H_2 may be observed via the dark fermentation. Apart from the expectation, the H_2 yield was very high since the pre-fermentative stage. The plenty of substrates may enhance the activities of acidogen and followed by the H_2 respiration of H_2 producing microbes, which could obtain the starch and glucose as substrates. The H_2 producing microbe at the dark fermentation was starve due to lack of glucose substrate. The few volume of H_2 was distributed by starch consuming H_2 producing microbe, nevertheless the H_2 producing microbes was dense in the dark fermentor. Irrespective of the two stages biohydrogen exhibited the behaviours of mixed and delective culture in the long term aging. The microbes appeared to be well adapt themselves to be survive and familiar with starch substrate. These preliminary experiments could be responsible for the reduction of substrate cost in biohydrogen process, if the starch was returned to be the glucose or short chain polysaccharide substrate via the pre-fermentative stage. The H_2 producing microbes at the dark fermentative stage could overwhelm the substrate inhibition, if the glucose or other primary substrate was increasingly added to the biosystem.