CHAPTER IV

RESULT AND DISCUSSION

4.1 Comparison test on heat treatment used in this study

The experimental result of the first set of batch test with different heat treatment conditions showed that the heat treated sludge of Boonrawd Brewery at 80-85° C for 30 minutes exhibited the highest hydrogen production with no methane gas. According to the comparison tests for validation of method between 3 kinds of seed sludge treatment at the reported condition, the result suggested that this method was an effective method for enrichment of H_2 producing bacteria; while simultaneously inhibiting the activity of methanogens.

4.2 Batch experiment

From the preliminary test result (Table 4-1), it was found that the test run No.1 when fed with solely brewery waste yeast (BWY) did not give any H_2 production. Because of only protein is a major constituent in the yeast cell which may lead to the unbalanced carbon/protein (C/N) ratio in the fermentation process. And H_2 is reported to be hardly produced from protein by several researchers (Noike and Misuno, 2000; Okamoto *et al.*, 2000; Lay *et al.*, 2003). The further work was thereafter carried out under various mixture of substrate except that of without glucose concentration. The batch experimental results of all 48 trials in phase I, investigated by using a central composite design (or CCD) are summarized in Table 4-2 and are illustrated as a response surface and contour plot with a point of maximum response in Figure 4-1. They indicated that 700 mg of glucose with 1,110.3 mg and 1,226.7 mg of brewery waste yeast (BWY) and heat-treated sludge respectively were optimal substrate mixtures for yielding the maximum H_2 production rate

(R_{max}) of 17.93 ml/h. At this point, additional confirmation test (Table 4-3 and Figure 4-2) was undertaken to check the statistically procedural accuracy. The experimental R_{max} was 18.76 ± 3.6 ml/h which was closed to that yield by RSM. This indicated that the estimated responses were fitted to the second-order polynomial model ($p<0.05$) employed in the study.

The second experiment in phase II was then conducted in a 1L CSTR using the optimal values obtained from the RSM to investigate H_2 producing fermentation. In order to compare efficiencies of the batch process operated with and without addition of BWY, two sets of batch experiments were conducted. The first set of batch experiment was run by using glucose and BWY at the optimal point, which were in equivalent to the concentrations of up to 7,000 mg/l and 11,103 mg/l (COD equivalent) respectively, as a substrate whereas the second set was operated with glucose of 7,000 mg/l alone as a substrate. For each set of the batch experiment, seed sludge of approximately 12,000 mg/l of VSS was used as inoculums. In the study biogas productions found containing only $H_{2(g)}$ and $CO_{2(g)}$, whereas $CH_{4(g)}$ was not detected in all experiments.

Table 4-1 The preliminary test data

Experimental No.	Seed (mg)	glucose (mg)	yeast (mg)	avg. Rm (mlH_2/h)
1	500	0	1500	0.0
2	500	500	500	4.3
3	500	1000	500	13.5
4	500	1000	500	17.3
5	500	1000	0	13.2
6	500	1000	0	12.1
7	500	1000	1500	62.3
8	500	1000	2000	2.4
9	1000	1000	0	21.0
10	1000	1000	500	15.7
11	1000	1000	1500	25.3
12	1000	1000	1500	24.5
13	1000	1500	2000	8.4
14	1000	1500	2000	7.0
15	1500	1000	1500	7.9
16	1500	1500	2000	9.0
17	2000	1000	1500	16.6
18	2000	1500	2000	8.6

Table 4-2 Full factorial central composite design with variables in coded and natural units along with the observed responses.

Run No.	X1	X2	X3	Seed (mg)	Glucose (mg)	yeast (mg)	Max. H_2 production rate (ml/h) STP			
							Rep.1	rep.2	rep.3	avg
1	-1	-1	-1	500	500	0	2.62	1.92	2.60	2.38
2	1	-1	-1	1500	500	0	5.98	6.35	4.88	5.74
3	-1	1	-1	500	1000	0	2.54	2.39	2.61	2.51
4	1	1	-1	1500	1000	0	7.94	6.87	5.79	6.87
5	-1	-1	1	500	500	1500	10.11	11.06	10.13	10.43
6	1	-1	1	1500	500	1500	18.80	15.71	15.21	16.57
7	-1	1	1	500	1000	1500	13.47	12.55	14.05	13.36
8	1	1	1	1500	1000	1500	17.91	18.47	21.94	19.44
9	-	0	0	159	750	750	3.39	3.55	3.20	3.38
10	1.682	0	0	1841	750	750	11.45	11.02	9.28	10.58
11	1.682	-	0	1000	329.5	750	4.90	5.08	6.12	5.37
12	0	1.682	0	1000	1170.5	750	6.95	7.11	5.62	6.56
13	0	1.682	-1.682	1000	750	0	3.18	3.03	3.43	3.21
14	0	0	1.682	1000	750	2011.5	6.13	4.74	5.06	5.31
15	0	0	0	1000	750	750	16.41			
16	0	0	0	1000	750	750	17.12			
17	0	0	0	1000	750	750	18.62			
18	0	0	0	1000	750	750	18.32			
19	0	0	0	1000	750	750	20.72			
20	0	0	0	1000	750	750	12.01			

Table 4-3 The result of confirmation experiment at optimal point

Run	VSS (mg)	Glucose (mg)	Yeast (mg)	H_2 Production Rate(ml/h) at STP
21	1226.73	700	1110.26	18.76±3.6

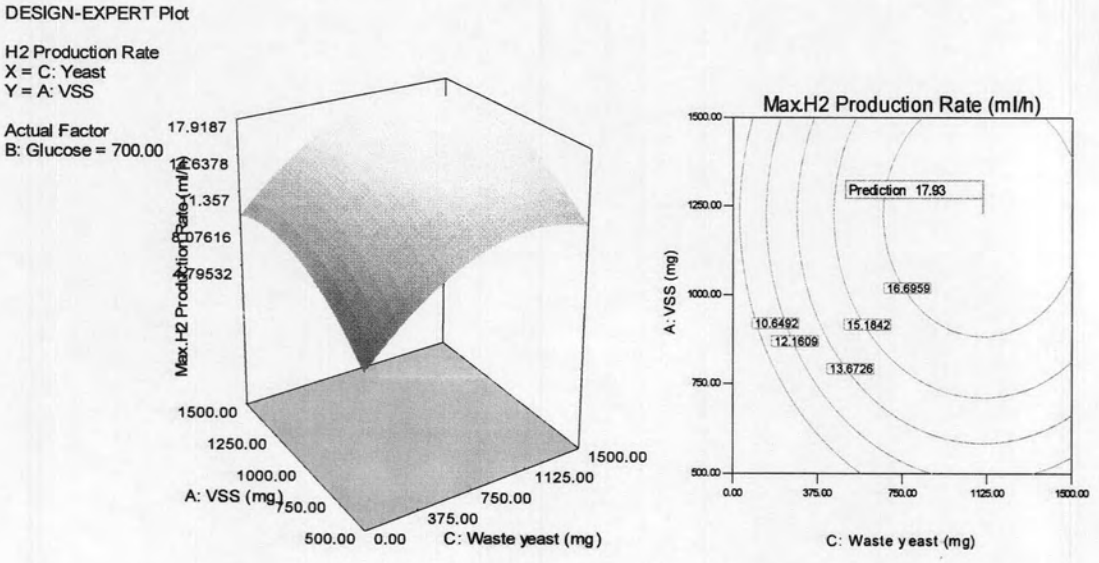


Figure 4-1 Response surface (left) and contour plot (right) illustrating a maximum hydrogen production rate (ml/h)

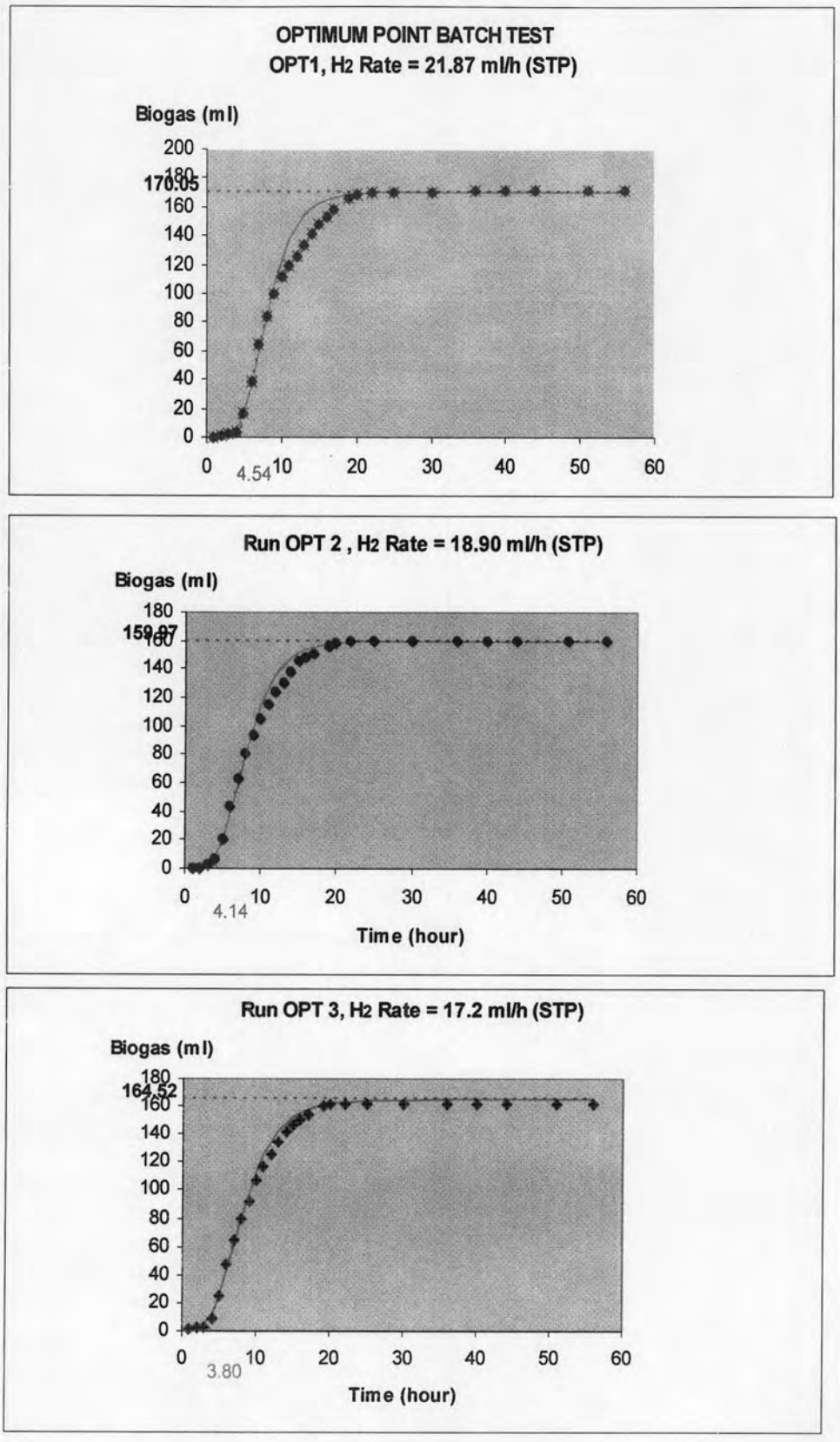


Figure 4-2 Cumulative hydrogen production at optimum point

4.3 Batch hydrogen production

The cumulative H_2 production curves obtained from the experiments were present in Figure 4-3 and Figure 4-4 respectively. It was shown that the observed data sets were fitted well to those estimated by the modified Gompertz model (Equation 1, Chapter 3) with the correlation coefficient (R^2) higher than 0.98. As start, both bioreactors operated with and without BWY adding were started–up immediately, within a few hours. Both cultures produced no methane. This implied that the heated mixed culture using in this study is considered usable as a seed in fermentation of larger scales.

During the batch operation, hydrogen yield and specific H_2 production rate (see Table 4-4) of 1.08 mol H_2/ mol glucose and 2.47 ml H_2/g VSS.h were observed in a CSTR operated without BWY. Whereas in the CSTR with BWY, the result were 1.42 mol H_2/ mol glucose and 3.90 ml H_2/ gVSS.h, respectively. Better H_2 yield and rate from CSTR with BWY was clearly obtained from yeast addition. As known commonly, yeast contains high nutritional values not only amino acids (see APPENDIX C) but also a rich sources of vitamin B-complex especially vitamin B1 (thiamine), vitamin B2 (riboflavin), provitamin D, nicotinic acid and biotin (Jean de Clack, 1957), which can induce a number of enzymes (Lee, 1966). It is presumed that BWY addition might stimulate spore germination of H_2 producing microorganisms, e.g. Clostridia strains (Doyle, 1989), might result in higher number of H_2 producing bacteria. Koku (2003) indicated that the use of yeast extract to replace vitamins could enhance the bacterial growth and hydrogen production. Angenent (2004) found that the increasing of H_2 yield followed the NADH pathway which is strongly dependent upon hydrogen partial pressure (P_{H_2}) in the reactor. Using CSTR in this study, might lower P_{H_2} and lower the reoxidizing of NADH, then resulting in the higher yield of H_2 per mol of glucose (or hexose). This yield seems to be relatively low as compared to the theoretical yield of 4

mol H_2 / mol glucose. However, improving more yield of H_2 can be done by means of several ways, including continuous feeding. Besides the rich vitamin could speed up the rate of biohydrogen production reaction by lowering the activation energy barrier (Figure 4-5) and thereby enhancing the rate of H_2 production (Wang *et al.*, 1979).

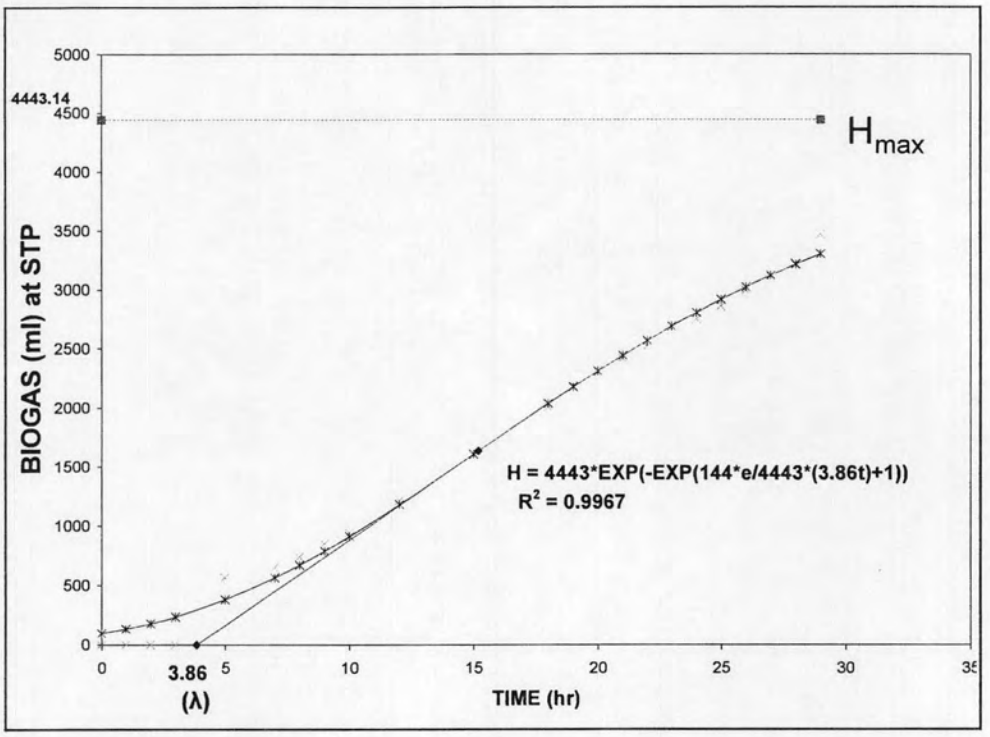


Figure 4-3 Cumulative biogas production curve in the CSTR batch without waste yeast adding

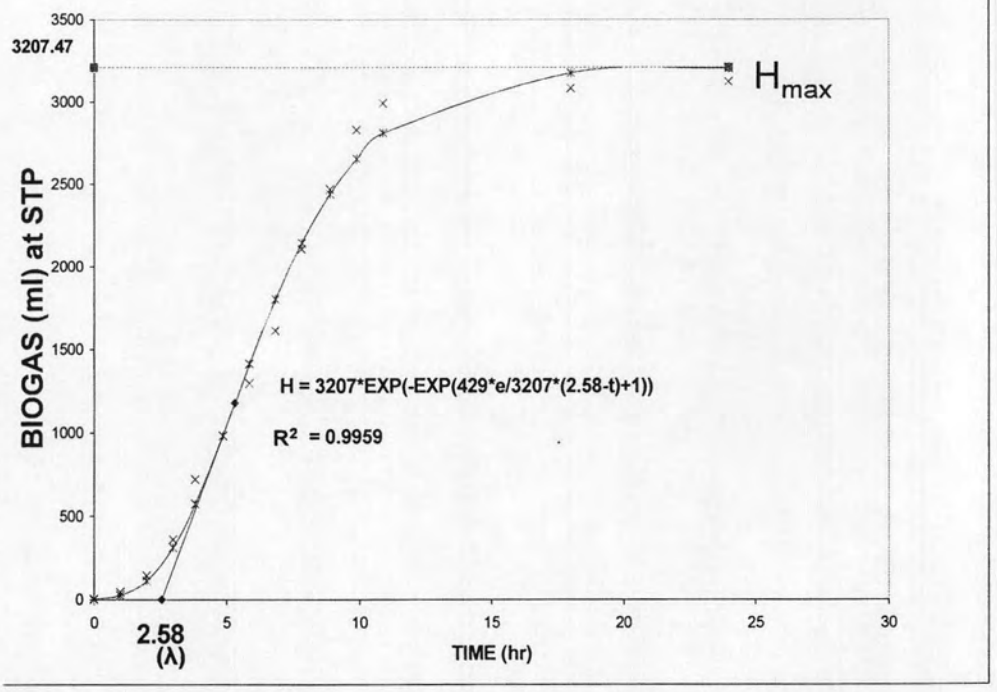


Figure 4-4 Cumulative biogas production curve in the CSTR batch with waste yeast adding

Table 4-4 Comparison of important parameters in batch culture (mean value)

Parameter	No-waste yeast	Waste yeast adding
maximum H_2 production rate (ml/h)	144	429
specific H_2 production rate (ml/gVSS.h)	2.47	3.90
% H_2	17.8-31.7	17.1-44.8
H_2 yield (mol/mol glucose)	1.08	1.42
The apparent kinetic value of k (d^{-1})	0.52	1.84
Soluble metabolites (mg/l)		
Acetic acid	14,076	25,265
n-Butyric acid	3073	1501
propionic acid	n.d.	32.7
ethanol	552	2135
% of theoretical yield *	24.6	32.3

*The theoretical maximum yield of H_2 fermentation via acetate pathway is 4 mol/mol glucose, corresponding to 0.0498 L H_2 /g glucose

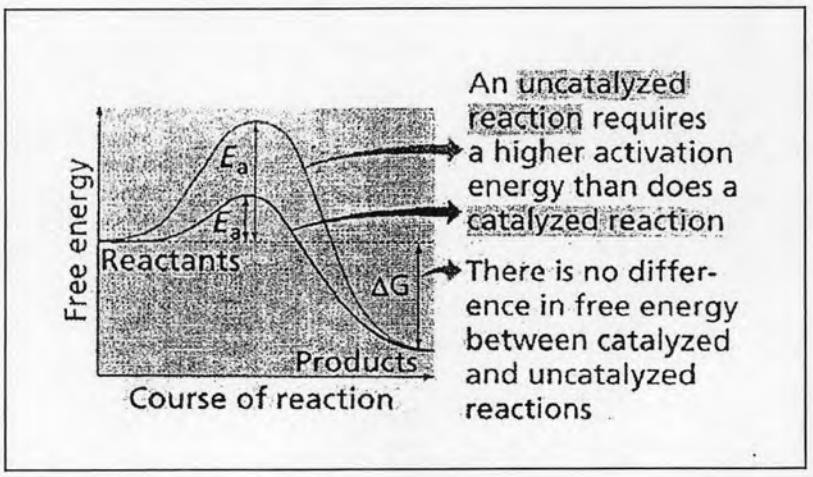
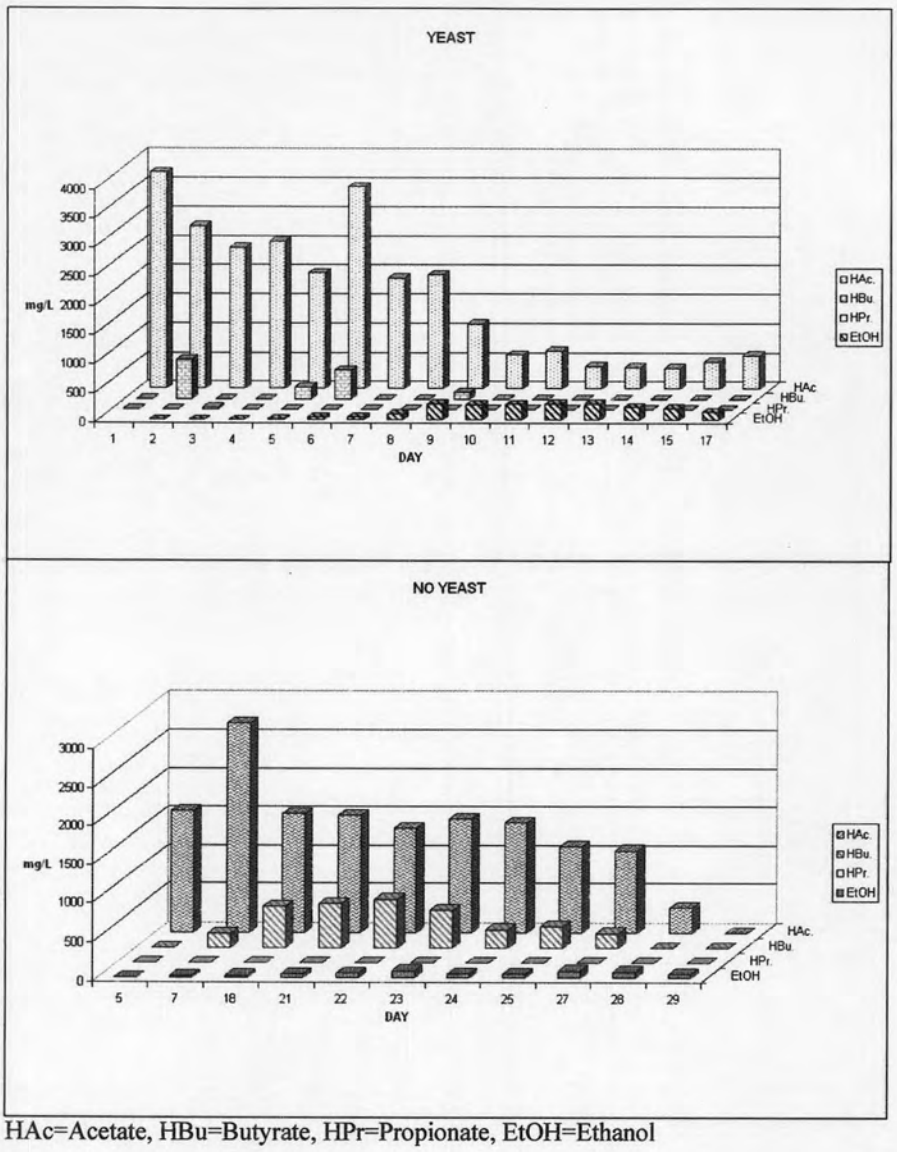


Figure 4-5 The use of enzymes can lower the activation energy of a reaction

4.4 Batch soluble metabolites

The fermentation and products obtained from both sets of batch experiment as summarized in Table 4-4, indicated that acetic acid and butyric acid were found as the major products while propionic acid was non – detectable. A small amount of ethanol was also found. Figure 4-6 shows the distribution of soluble metabolites for duration of the batch experiment. Acetic acid was observed as the principal products (87%) in the culture with BWY. While only 79% was obtained from the batch without BWY. These observations reflect the increase in hydrogen yield as mentioned before. Similar results were obtained using some physical techniques (e.g., N_2 sparging or vigorous stirring) (Mizuno *et al.*, 2000 and Lamed *et al.*, 1988). The addition of waste yeast might influence acetate formation regarding the maximized H_2 production. The four moles per one mole of glucose could be achieved if all substrate was converted to acetic acid (Thauer, 1977).

The overall yield obtained from this study were only 1.08 and 1.42 H_2 produced per mol of glucose, correspondingly to the conversion efficiency of 24.64% and 32.25%, respectively. This could be due to the natural characteristic of batch cultivation. The long retention time required to reach a stationary phase, would shifted metabolic pathway towards rapid solvent production such as lactate, ethanol, acetone, butanol, or alanine (Levin *et al.*, 2004).



HAc=Acetate, HBu=Butyrate, HPr=Propionate, EtOH=Ethanol

Figure 4-6 The concentration of VFAs and ethanol in the CSTR batch experiment

4.5 The kinetic parameter

The Monod kinetic parameters of k and Ks were investigated by fitting batch CSTR experimental results to integrated Monod model with R^2 greater than 0.98 (Table D-1, Table D-2 in APPENDIX D). The estimated results obtained from both sets of batch experiments were shown in Table 4-4. It was shown that the apparent kinetic value of k revealed a significant change in the maximum rate of glucose utilization. In this study, Ks represents the substrate affinity of microorganisms obtained in nearly as the same value (approximately 2.3 g/l) from both batches. Thereby, in according to the shifted k value (Table 4-4), it confirmed effect of BWY addition on hydrogen production.

4.6 Continuous feeding experiment

The CSTR configurations, shown in Figure 3-5 and 3-6, was used in the continuous mode experiment. The reactor was designed under a concept of separating between HRT and SRT values. The microbial biomass generated from the process was separated and returned to the complete-mixed reactor which allowed long SRTs to be maintained with very shortening HRT operation in analogous to the activated sludge process with recycling system. Thus a basic parameter used for design and operation was the solids retention time (SRT), representing the average time durations during which microbial biomass has remained in the system. The SRT determination was calculated using the equation 2-5. Due to a short-term batch test, the decay coefficient (K_d) was assumed to be insignificant and was negligible and the growth yield coefficient (Y), obtained from Table 4-5, was 0.1 g/g.d. By rearranging Equation 2- 5 and substitute the Monod kinetic parameter values of k and Ks (from Table 4-4) of 1.84 d^{-1} and 2.357 mg/l, respectively.

The SRT could be computed as shown below:

$$SRT_{minimum} = \frac{[0.1\ (g/g.d)*\ 1.84\ (d\text{-}1)\ *\ 7.0\ (g/l)]^{-1}}{2.357\ (g/l) + 7.0\ (g/l)}$$

$$= 7.265\ d$$

$$\text{Design SRT} = 1.5(7.265) = 10.9\ d$$

Value of 1.5 is given as safety factor used in the design of SRT which allowed flexibility for operational variations in anaerobic treatment processes. As recommended by Metcalf and Eddy (2004), at 30°C the SRT should be greater than 20 d for effective anaerobic treatment process. Therefore the shortest HRT reduction could be done over the SRT ranging between 10 – 20 d, unless otherwise the process failure could be obtained. To determine the SRT during the operational runs, the equation below was used:

$$SRT = \frac{VXo}{QXe} = \frac{(HRT)*Xo}{Xe}$$

Where
V = reactor volume (L)
Q = influent flow rate (L/d)
Xo = concentration of biomass in the reactor (g/L)
Xe = concentration of biomass in the effluent (g/L)

Table 4-5 Parameters for completely mixed suspended growth reactors treating soluble COD

Parameters	Unit	Value	
		Range	Typical
Solids yield,Y			
Fermentation	gVSS/gCOD	0.06-0.12	0.10
Methannogenesis	gVSS/gCOD	0.02-0.06	0.04
Overall combined	gVSS/gCOD	0.05-0.10	0.08

Source: Metcalf and Eddy, 2004 (p.1000)

4.7 Effect of HRT on hydrogen production

The effect of HRT on H_2 production was studied in a CSTR operated at pH and temperature of 5.0 and 30°C respectively including other parameters that was investigated to identify boundary conditions for stable continuous process before in the batch experiment. As started-up, HRT of 40 h was designated to the CSTR run. The system could be viewed as a fast start-up reactor by observation the produced biogas once the substrate was fed to the reactor and the efficiency of glucose conversion measured was 100 % within 24 hour period. The feeding mode was then switched to 24 h HRT on the next day. This active biomass may be attributed to a well-adapted sludge taken from the large-scale UASB reactor which acclimate to the brewery waste yeast mixing in the wastewater for extended periods, and confirmed the effectiveness of heat treatment protocol used in this experiment. Table 4-6 summarizes the result of reactor performance during the continuous flow experiment (the raw data was tabulated in Table D-3, APPENDIX D). Figure 4-7 to 4-11 illustrates the dynamic manner of H_2 production investigated during the time interval of HRT variation. Based on the results, this reactor took about 4-5 days to reach steady-state condition (based on ± 10% the produced biogas variation). After reaching steady-state, HRT was decreased in steps reducing from 24 to 4 hours. At each run of HRT, the reactor was maintained at least for 10 days before transition to new HRT. The H_2 productivity was all increased as the HRT decreasing and no methane was detected in the biogas produced. A sudden increase in the H_2 yield was noticed when the HRT was shortened to 4 hour (Figure 4-7). Similar pattern was also observed on the overall yield of H_2 production H_2 (Figure 4-8 to figure 4-11). During this time, the observed cell washed-out, representing as the value of effluent SS was kept low (Figure 4-12), while the reactor SRT was still higher than the SRT_{min} (approx. 10 day) value (Figure 4-13). The resulting biomass retained in the reactor as shown in Figure 4-14 is

ranging between 5764.67 to 9540.32 mg/l during the entire period of HRT variation. This is likely to be the configuration of the reactor CSTR used in this study.

As shown in Figure 4-15, although the glucose conversion rate is sharply dropped to about 20%, it turns out that the H_2 yield, at steady-state, was obtained at the maximum value ranging from 2.37-3.82 mol/mol glucose utilized. This may be due to shock load effect which will be discussed in greater details on the following section. It is interested to note that this reactor performance is focused on the H_2 production irrespective of efficiency treatment, analogous to a hydrolysis tank of anaerobic digestion. The metabolite by-products, which were mostly composed of VFAs will thereupon be converted to CH_4 or H_2 by further fermentation.

Table 4-6 Result of reactor performance during the continuous flow experiment (mean value)

Parameter	unit	HRT Variation (h)			
		24 (day1-10)	**12** (day11-20)	**8** (day21-30)	**4** (day31-40)
VSS	mg/l	9540.32	8504.67	5903.67	5764.67
OLR	g glucose/l	7	14	21	42
Biogas production	ml/d	1301.15	5575.16	8450.00	10348.94
H_2 production	ml/d	519.10	2689.90	4826.95	6267.14
H_2	%	39.08	47.89	57.12	60.58
H_2 yield	mol/mol glucose	0.28	0.75	1.04	2.87
SHPR	ml H_2/gVSS.h	4.38	21.07	46.01	57.53
VHPR	l/l reactor.d	0.40	2.07	3.72	4.87
Effluent glucose	mg/l	0	124.71	1050.00	5562.8
Glucose conversion	%	100	98.22	84.94	20.53
SRT	d	130.84	25.12	17.43	16.38
Effluent SS	mg/l	74.14	174.00	116.25	58.80
Soluble metabolites					
Acetate	mg/l	40.65	456.78	1540.85	2034.31
Butyrate	mg/l	348.97	415.82	1305.28	1805.16
Propionate	mg/l	180.70	252.96	429.90	724.24

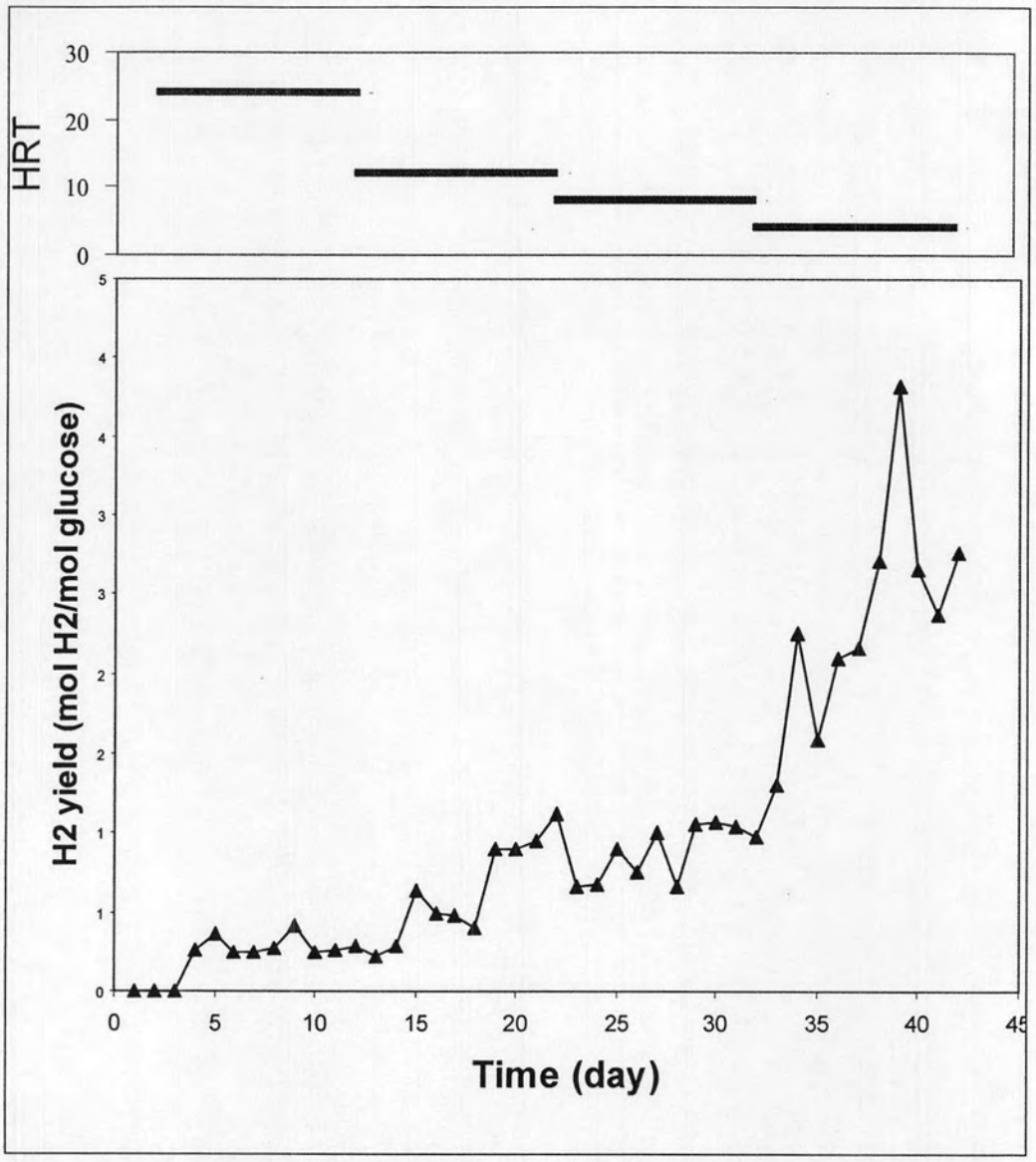


Figure 4-7 The effect of HRT on H_2 yield

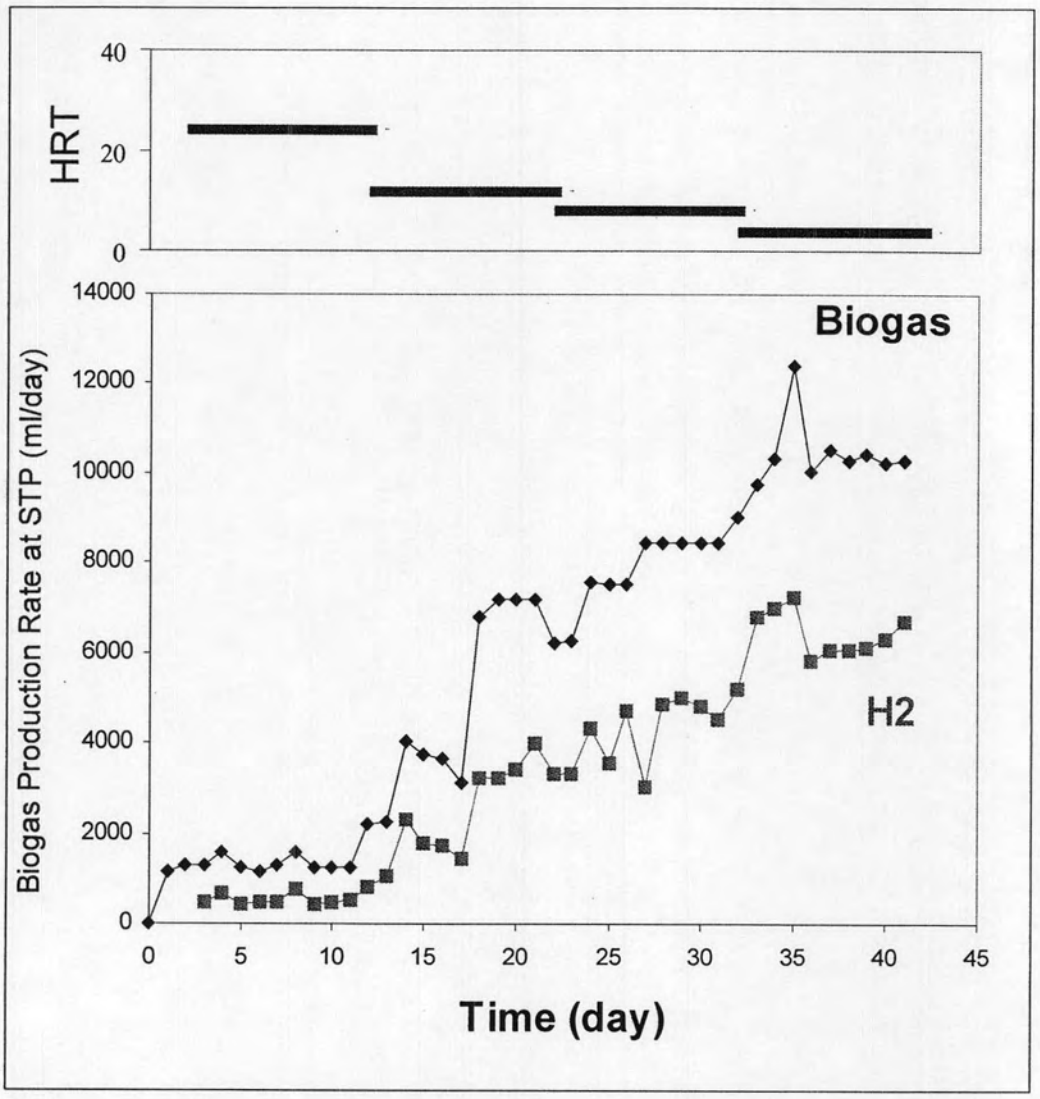


Figure 4-8 The effect of HRT on H_2 production

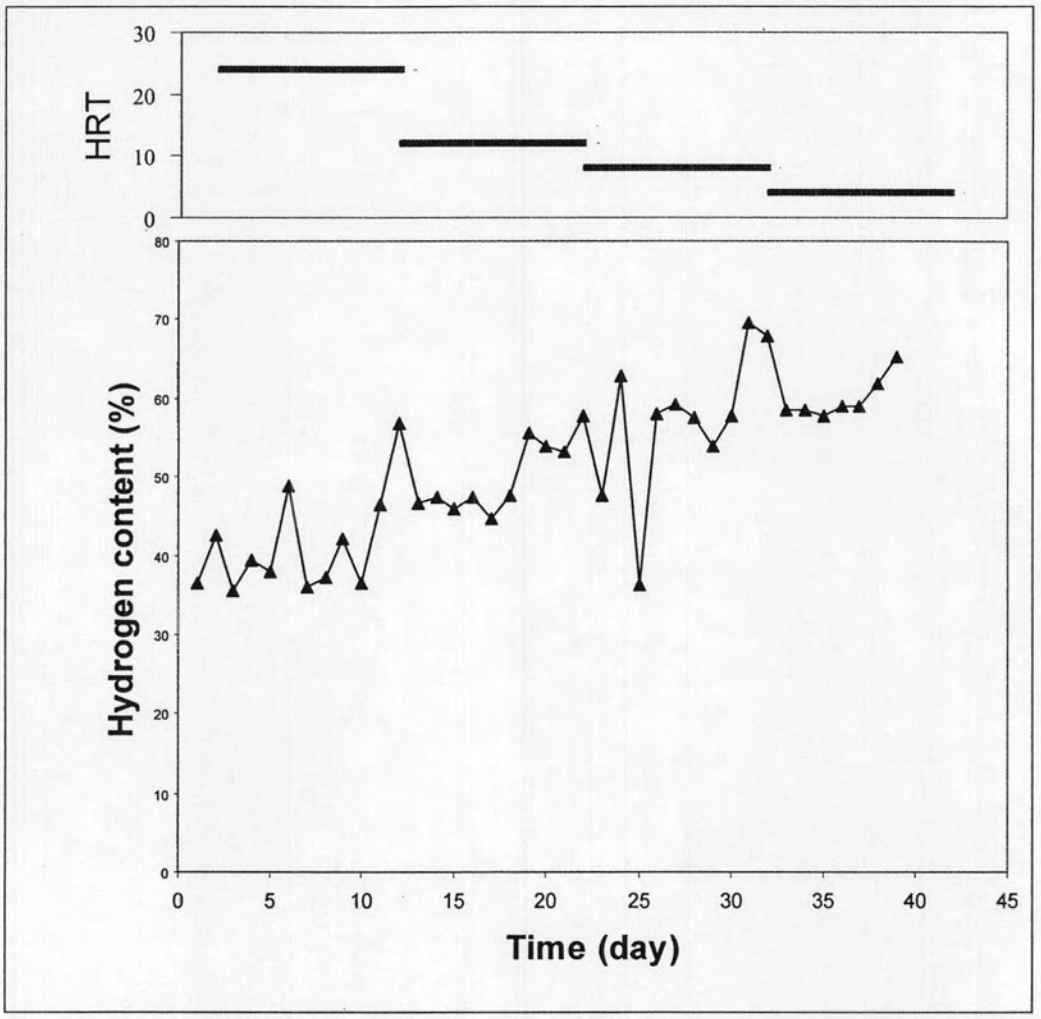


Figure 4-9 The effect of HRT on H_2 content

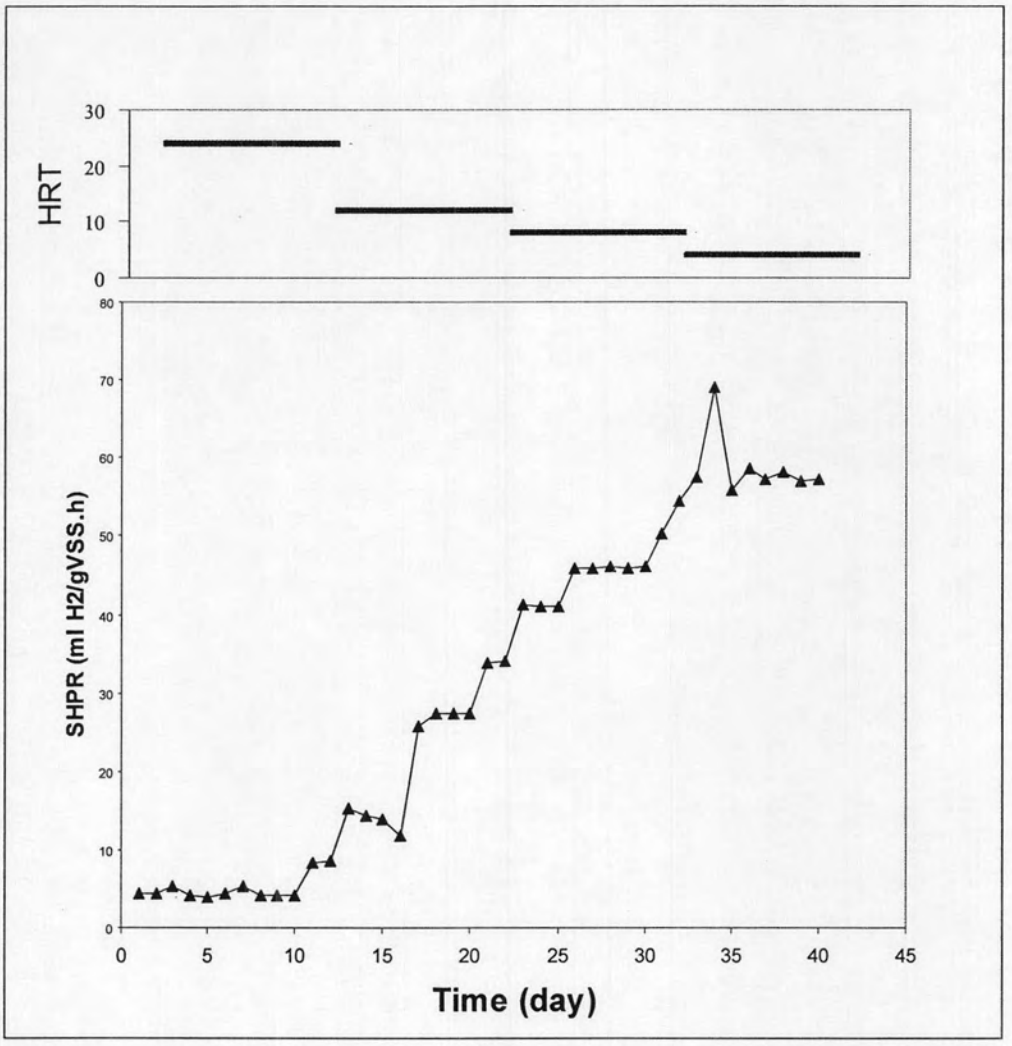


Figure 4-10 The effect of HRT on specific hydrogen production rate (SHPR)

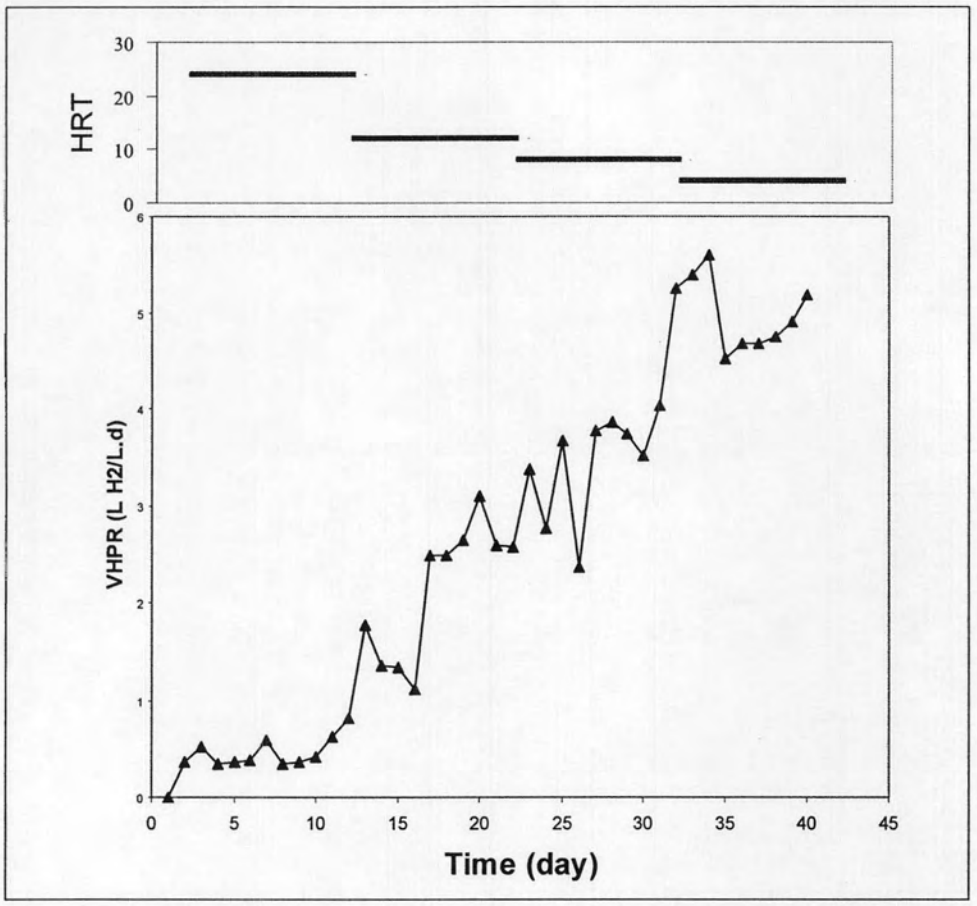


Figure 4-11 The effect of HRT on volumetric H_2 production rate (VHPR)

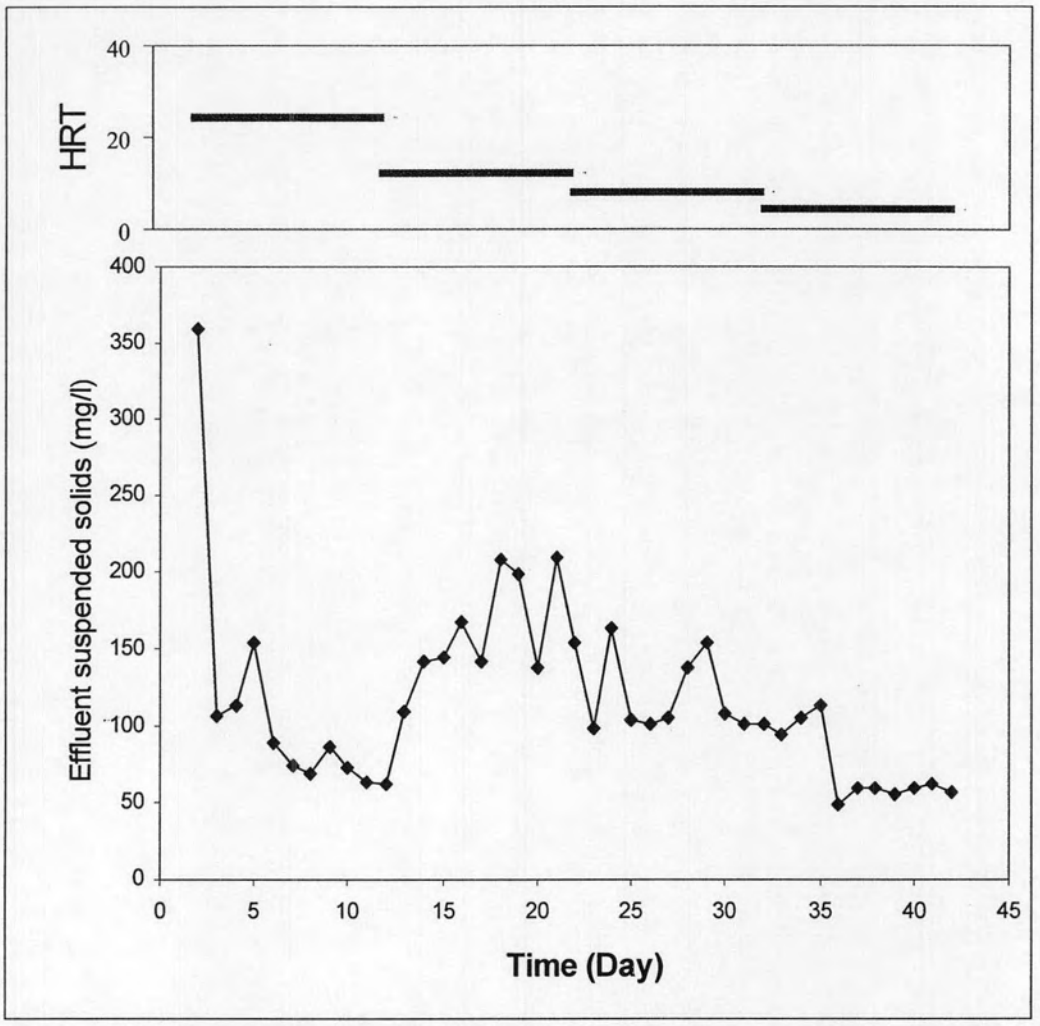


Figure 4-12 The effect of HRT on Effluent suspended solids (SS)

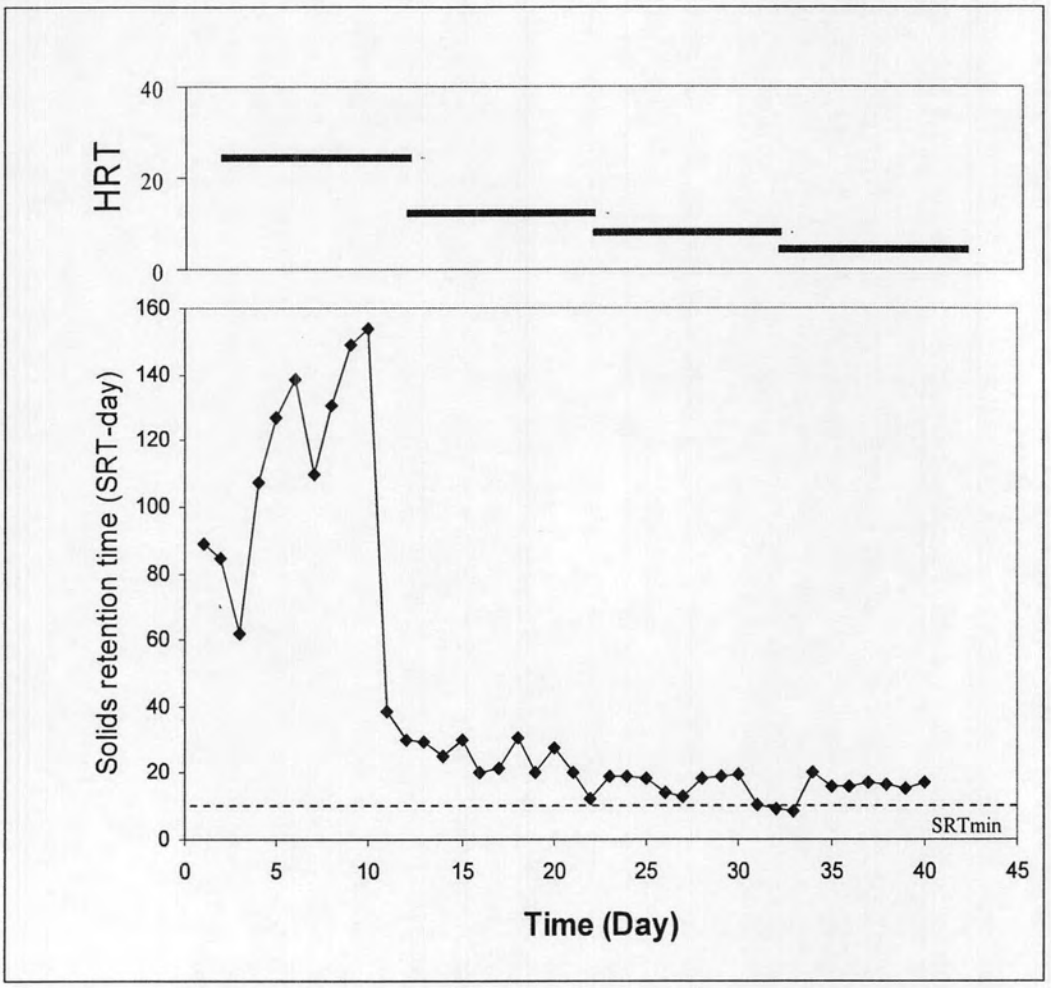


Figure 4-13 The effect of HRT on solids retention time (SRT)

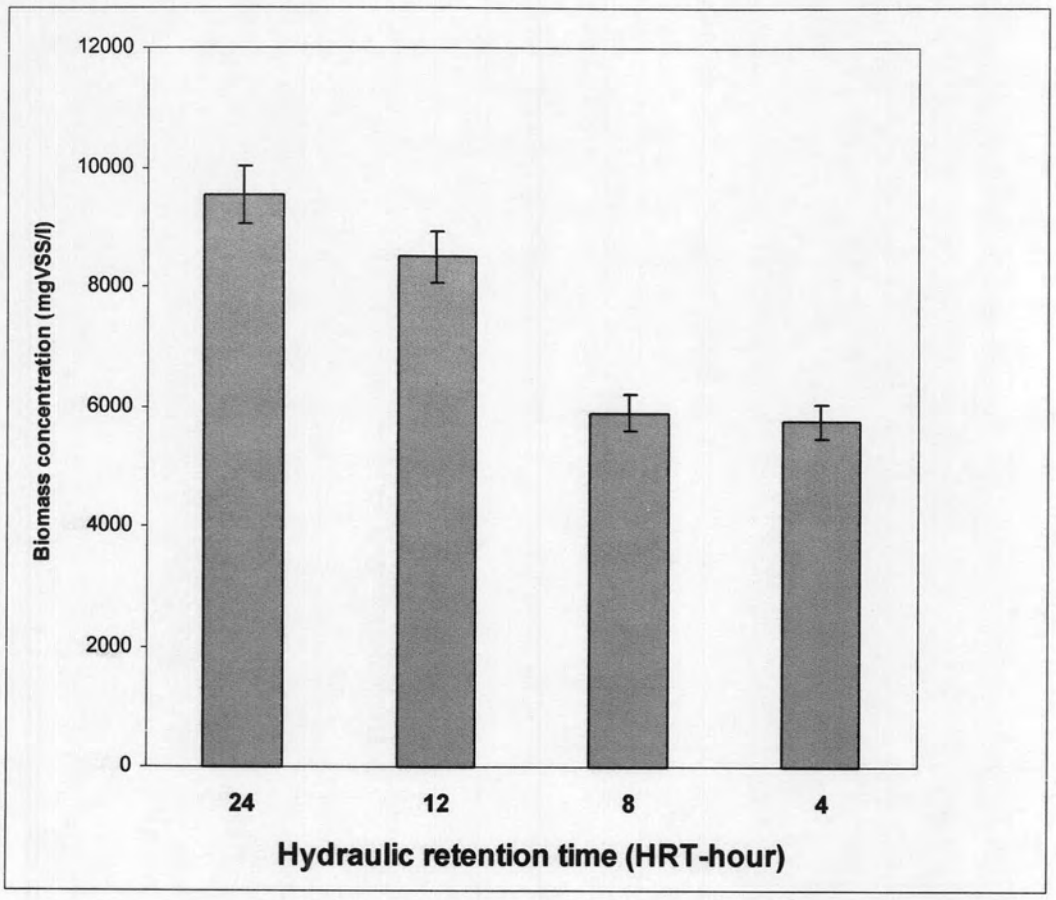


Figure 4-14 Hydraulic retention time and biomass concentration

4.8 Effect of shock load

The shock loading, by definition is of the reactor operated at high influent substrate concentration or higher organic loading. At HRT of 4 hour, corresponding to organic loading rate (OLR) as high as 42 g glucose/l.d, H_2 yield of 2.87 mol/mol glucose utilized (in average) was obtained with the highest % H_2 and total H_2 productivity as shown in Figure 4-16 to 4-20. The H_2 yield of 2.87 mol/mol glucose is considered the highest in comparison to those reported in the literatures for both pure and mixed cultures on glucose studies using various types of reactor. The range of 1.1-2.6 mol/mol of glucose was reported as shown in Table 4-7. The highest H_2 yield of 2.6 mol/mol glucose was reported so far by continuous operation on a sugary industrial wastewater using *Clostridium* (Taguchi *et al.*, 1996). Similar result was also reported that shock load also effect SHPR and VHPR as shown in Figure 4-19 and Figure 4-20 respectively. Further reduction was done but sludge wash-out was noticed when HRT was less than 4 hour, as a results of high turbulence occurred in the reactor.

Resembling to the well understood fed-batch operations in industrial fermentation scale, substrate is normally fed into the reactor in order to always maintain exponential growth phase of microorganisms (Bailey and Ollis, 1986). As the results the maximum H_2 production could be obtained. In the experiment, substrate was fed into the reactor after 2.6 hour, which was expected to be the exponential growth phase of microorganisms as shown in Figure 4-4. It is possible that extremely high substrate fed continuously during this period could keep the process to proceed with high production of H_2. Chen *et al.* (2006) suggested that the higher influent substrate concentration or organic loading rate (low HRTs), the higher H_2 production observed in continuous H_2 bioreactor. Moreover, the high volumetric flow rate, represented as short HRT, might contribute to rapid decrease of both P_{H_2} and unionized VFAs dissolved in the reactor that inhibits activity of H_2 producing bacteria. This also helps to

control recovery of methanogen in the long run continuous H_2 fermentation (Cha and Noike, 1997; Lin and Chang, 2004; Zhen *et al.,* 2006). This is similar to that reported by Khanal *et at.,* 2006, that the sequence batch reactor (SBR) operating with "feast and fast" mode might favor H_2 spore germination and also flushed-out inhibitory unionized acids. Therefore, the improvement of continuous hydrogen producing reactor could be done by shortening the HRT coincide with BWY addition strategy.

Table 4-7 H_2 yields and content in biogas from various reactor by dark fermentations

Organism	Carbon source	H_2 yields	%H_2 Content	Reactor/ HRT (h)	Reference
E. cloacae IIT-BT 08	Glucose (1%)	2.2 mol/mol glucose		Batch	Kumar and Das (2000)
Sludge compost	Glucose (10g/L)	2.1 mol/mol glucose		Batch	Morimoto *et al.*, 2004
Mixed culture	Glucose (20gCOD/L)	1.1 mol/mol glucose		CSTR /4h	Chen and Lin (2000)
Mixed culture	Sucrose (13.7 g/L)	1.2 mol/mol glucose	60	Trickling Biofilter/4-12h	Oh (2004)
Mixed culture	Glucose (7.0g/L)	2.1 mol/mol glucose	64	CSTR /6h	Fang and Liu (2002)
Clostridia sp.	Glucose (20 g/L)	1.7 mol/mol glucose	42.6	CSTR /6h	Lin and Chang (2004)
C. acetobutyrecum	glucose	2.0 mol/mol glucose	50	Fed-batch	Chin (2003)
Mixed culture	Sucrose (20g COD/L)	2.6 mol/mol glucose	35	SBR/4-12h	Lin and Jo (2003)
E. aerogenes	Starch (20g glucose/L)	1.09 mol/mol glucose			
E. butyrecum+*E. Aerogenes*	Starch (2%)	2.6 mol/mol glucose		Immobilized/0.75h	Yakoi *et al.*, 1998
Mixed culture	Sugar beet juice	1.7 mol H_2/ mol hexose		Batch	Hussy *et al.*, 2005

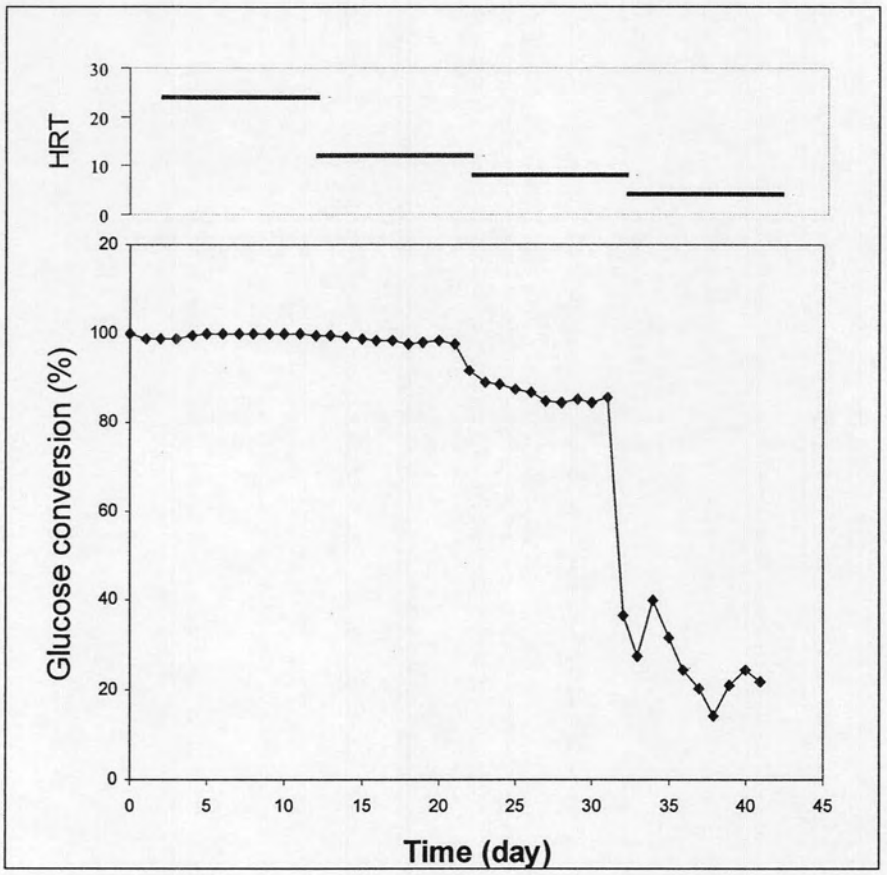


Figure 4-15 The effect of HRT on glucose conversion

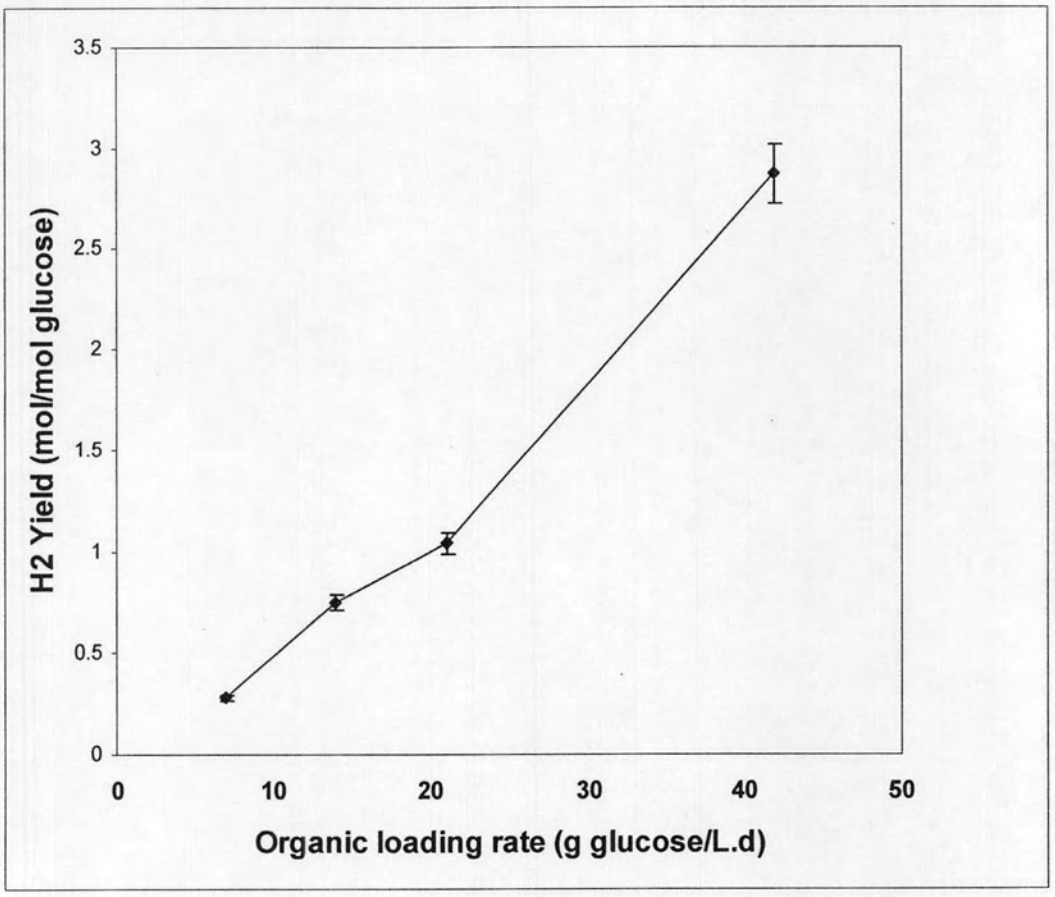


Figure 4-16 Organic loading rate and H_2 Yield

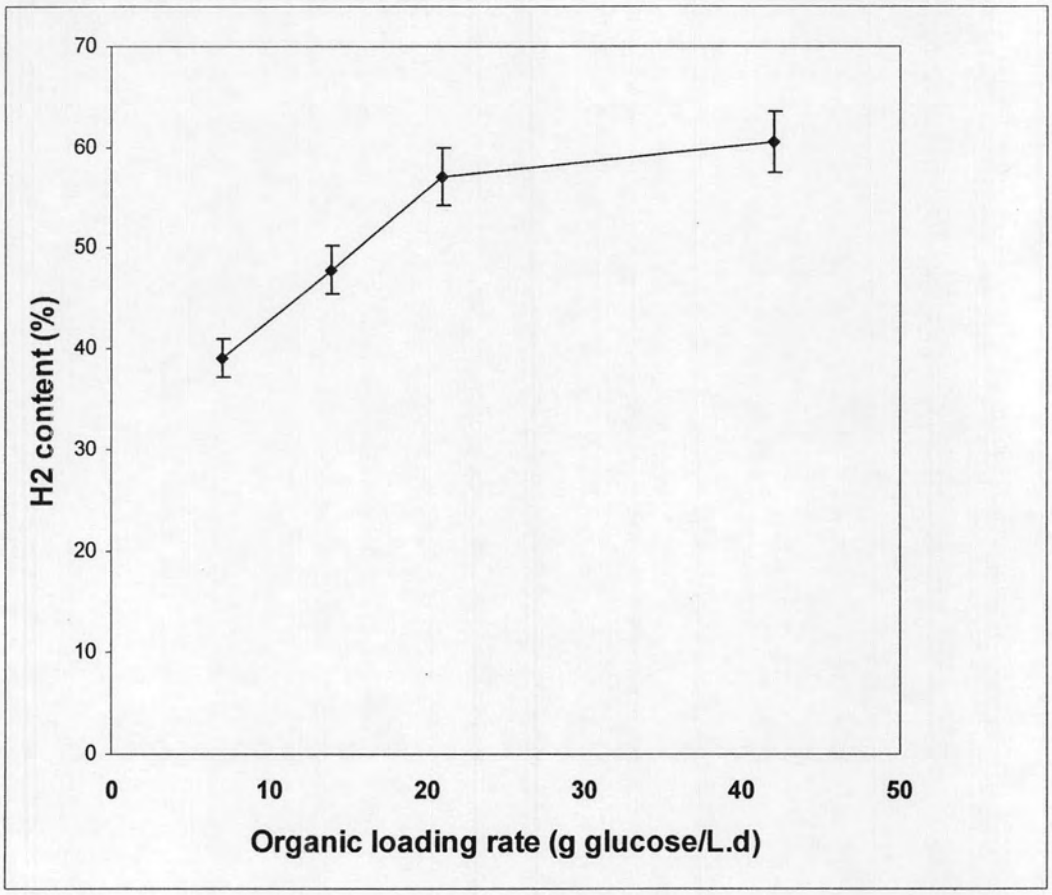


Figure 4-17 Organic loading rate and H_2 content

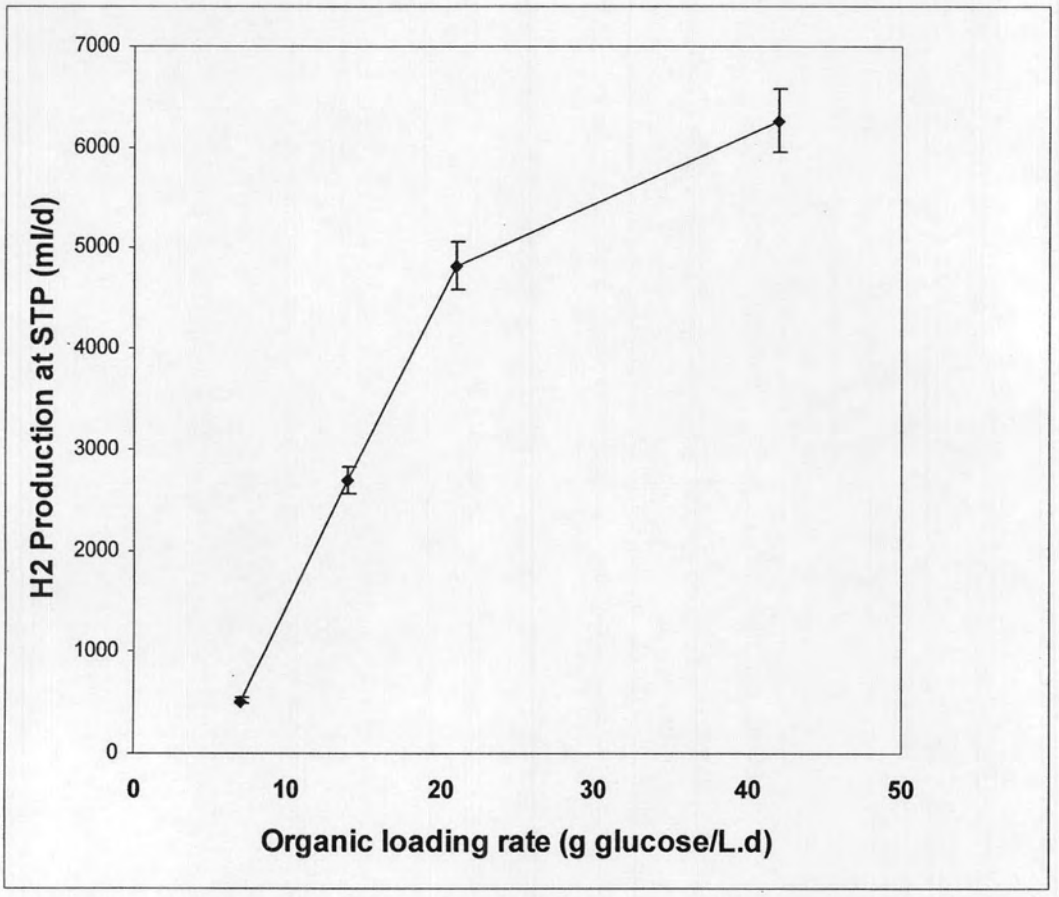


Figure 4-18 Organic loading rate and H_2 Production at STP

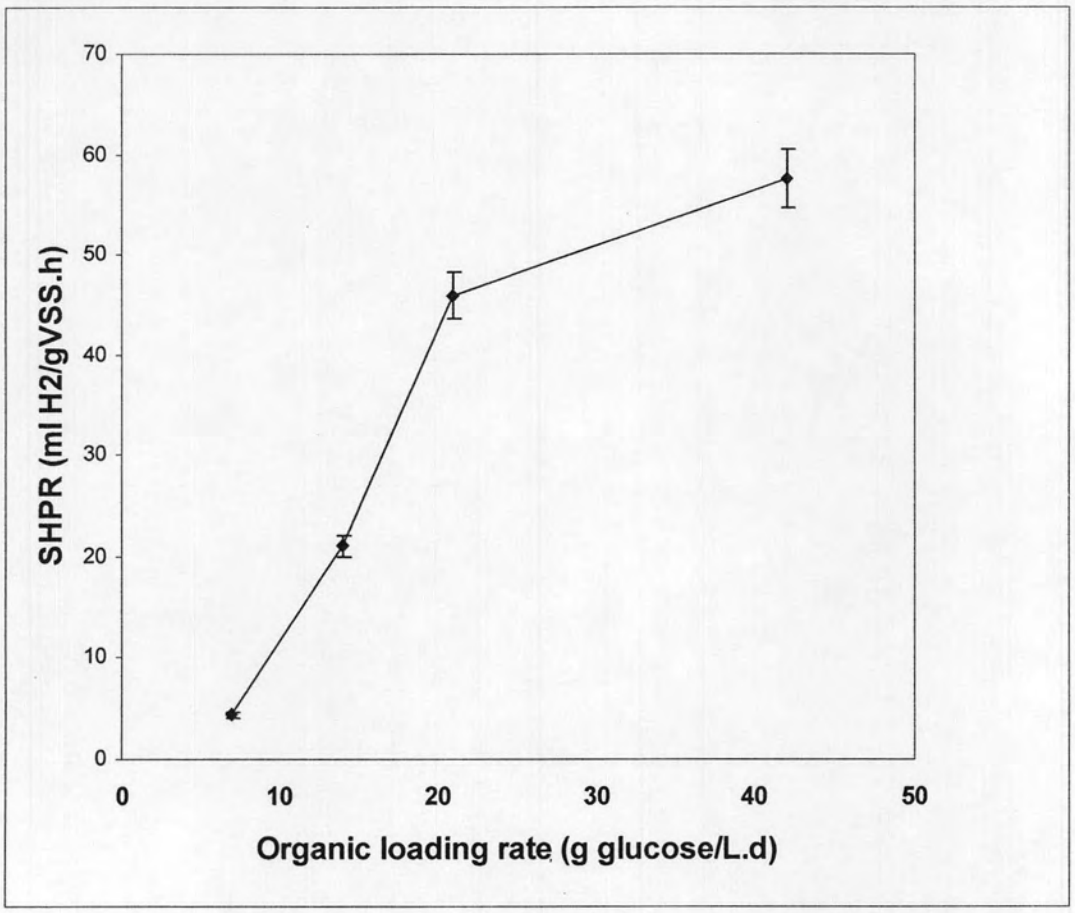


Figure 4-19 Organic loading rate and specific hydrogen production rate (SHPR)

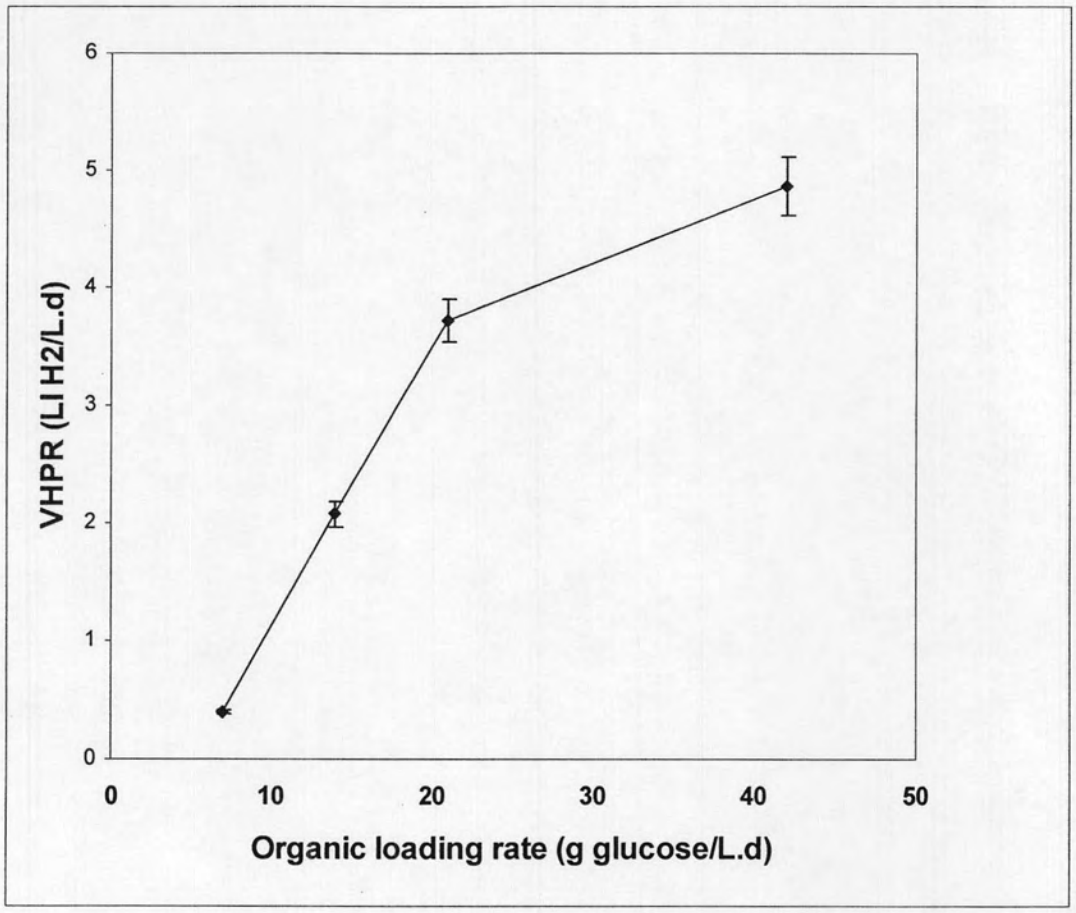


Figure 4-20 Organic loading rate and volumetric hydrogen production rate (VHPR)

4.9 Variation of VFA concentrations

The formation of hydrogen by dark fermentative bacteria, facilitated by hydrogenase, always involves volatile fatty acids (VFAs) and other metabolic products (Oh *et al.*,2006). The distribution pattern of soluble metabolites during the variation of HRTs as illustrated in Figure 4-21, shows that acetic acid (HAc), butyric acid (HBu) and propionic acid (HPr) were detected in all HRTs variations. The tendency of all VFAs concentration seems to increase with the decreasing HRT, while ethanol was non-detectable. Nevertheless overall yield of H_2 production was higher following the decreased HRTs. The acetic and butyric acids were the major metabolites which accounted for 87% of total VFAs. Meanwhile the H_2 production, H_2 yield, % H_2, specific H_2 production rate (SHPR) and volumetric H_2 production rate (VHPR) were adversely obtained in higher values (as tabulated in Table 4-6) during the 4 h HRT operation, compared to those obtained in the other HRTs. These results reveal that in the presence of propionic acid, quantity of H_2 formation may not be effected. In contrary to those reported by several researchers, the propionic production was always found in a small quantity in the continuous reactors running at low HRTs ranging between 4-12 hours (Cha and Noike, 1997; Hussy *et al,* 2003; Lin and Chang, 2004). These results indicate that the mechanical dilution did not effect on the selection of propionate producers in the mixed culture used in this study. This presumably due to at 4-8 hour HRT, a short retention time may not be sufficiently long enough to allow H_2 microorganisms converting H_2 and VFAs to reduced fermentation end products such as lactate or alcohol. As the results, it correspondingly has higher H_2 productivity and VFAs (Levin *et al.*, 2004). In addition, the butyrate/acetate (B/A) or acetate/butyrate (A/B) ratio at each steady-state condition (Table 4-8) did not show a certain proportional to H_2 yields. Thus it was not used as an indicator for improving H_2 production. This probably is due to the continuous H_2 fermentation which is attributed to different factors such as substrate, inoculum, heat treatment protocol and also

other operational conditions e.g. pH and temperature (Fang and Liu,2002; Lin and Chang; 2004). So far the experimental results in respecting to VFAs were reported differently in the relevant literatures (Hawkes *et al*., 2007).

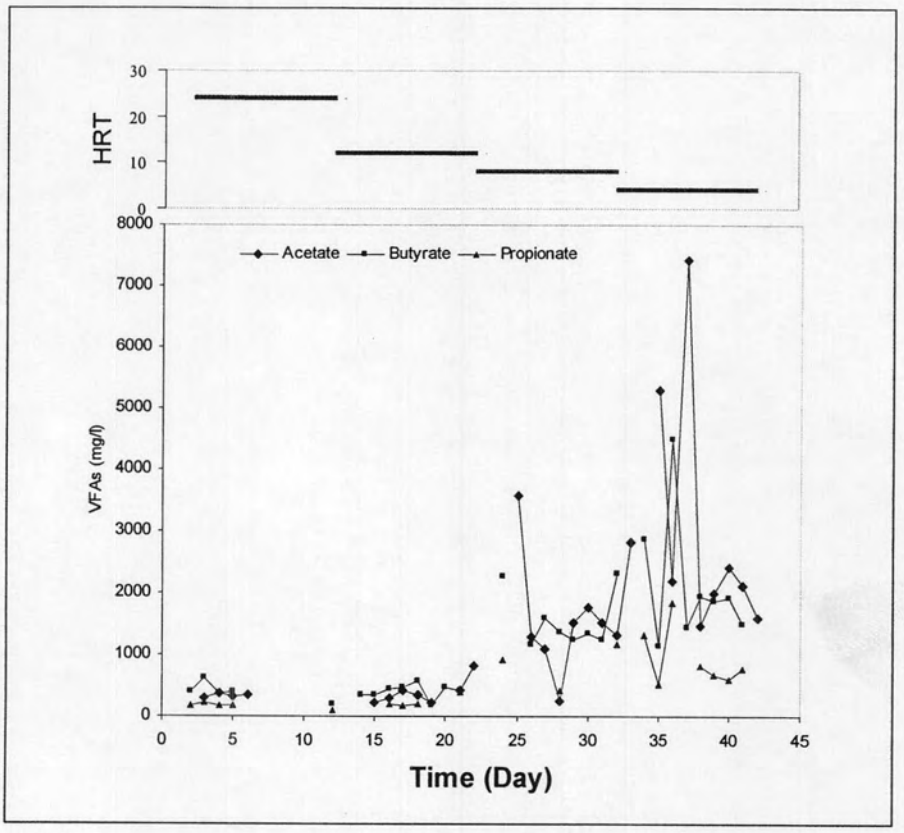


Figure 4-21 The effect of HRT on volatile fatty acids (VFAs)

Table 4-8 The average value of volatile fatty acid (VFA) production at different HRTs

HRT	VFAs	Constituents (%)			B/A	A/B
(h)	(mg/L)	Acetate	Butyrate	Propionate	ratio	ratio
24	870.37	39.14	40.09	20.77	0.70	1.43
12	1125.56	40.58	36.94	22.47	0.62	1.61
8	3275.22	47.04	39.85	13.10	0.58	1.73
4	4563.71	44.58	39.55	15.87	0.60	1.65

Remark: B/A and A/B ratio based on molar basis

4.10 Microbial diversity at different HRT

In order to identify the impact of HRT on the microbial community, total DNA extractions were prepared from each different HRT which were analyzed and compared using PCR-DGGE techniques with the DGGE profile of 16S rDNA gene fragments as shown in Figure 4-22. The comparisons of partial 16S rDNA cloning sequences to high similarity sequences are shown in Table 4-9. Each band on the DGGE profile corresponded to a gene fragment of unique 16S rDNA sequences and accordingly represented a specific species in the microbial community. The intensity of a band represents a relative abundance of the corresponding microbial species. The DGGE profile is clearly shown that the seed sludge sampling at day 0 (Table 4-10), the majority of microbial population are involved in H_2 fermentation which is consistently to those predictable result obtained in the section 4.2 dealing with the BWY addition enhancing the amount of H_2 producing bacteria. As we have known from the section 2.3.1, Chapter 2 that the hydrogen-producing metabolism depends on the activity of several enzymes such as pyruvate:ferredoxin oxidoreductase (PFOR), ferredoxin, and hydrogenase, which are either an iron containing or iron-sulfur containing enzymes directly responsible for H_2 formation (Ljungdahl *et al.*, 2003). Thus, apart from nitrogen (N) and phosphorus (P), nutrients known to be significant anaerobic bacterial growth, iron (Fe) and sulfur (S) containing BWY might be another component in hydrogen

producing enzymes. The sludge samples from different steady-state representing as The DGGE-profile also shows a shift of microbial population with HRTs (Figure 4-22). An apparent intense band, represents the dominant species on each lane which was observed at each HRT after reactor start-up as compared to the seed sludge sampling at day 0 (Table 4-10). The microbial diversity decreased as the HRT was shorten to 8 hour as can be shown by disappearing of Band A, B and C, while only Band D and E still remained during 8h and 4h HRT, respectively. This seems to be due to the wash out effect of higher feeding rate (low HRT). Though, at very high hydraulic flow rate (4h HRT) the dominant species of Band D and E (lane no. 5) turned out and still retained in the reactor which existed in a more abundance as shown in a more intensive band compared to those of lane no. 4, along with the maximized hydrogen yield. This result reveal that the dominant species (see Table 4-9) responsible for the hydrogen production, acetate, butyrate and propionate were affiliated with the uncultured clone HPR 146 and *Clostridium pasteurianum* of which commonly known as a key role on H_2 fermentation (Tagushi *et al.*, 1992). In summary, shortening of the HRT to 4 h enables reducing of the diversity of microbial population which is associated with an elimination of undesirable microorganisms without affecting the existence of dominant species. This was the presumable reason for the observed increase in H_2 yield. It might be concluded that the shift H_2 yield from 1.42 mol/mol glucose (in the batch test) to 2.87 mol/mol glucose (in the continuous flow CSTR operated at 4h HRT) resulted from the contribution of HRT of the selected microbial population (Fan *et al.*, 2006; Chang *et al.*, 2007; Zhang *et al.*, 2006).

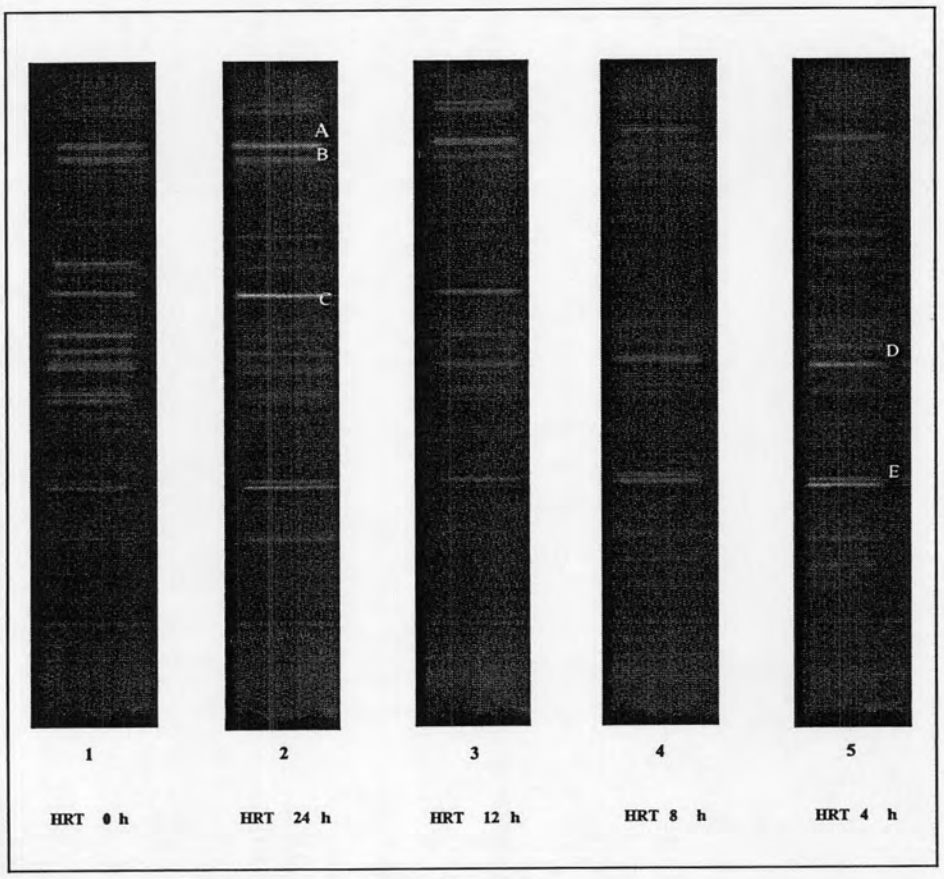


Figure 4-22 Variation of DGGE profiles with HRTs

Table 4-9 Similarity of sequences obtained from excised DGGE bands to sequences in the NCBI database

Band	Closest species in GenBank	GenBank accession	% Similarity	Source
A	Uncultured bacterium Clone HPR 124	DQ 464581.1	98	H_2 fermentor
B	Unculture bacterium Clone HPR 124	DQ 464581.1	98	H_2 fermentor
C	*Pectinatus frisingenesis* Isolate R 54-28	EU 589446.1	98	H_2 fermentor
D	*Clostridium pasteurianum* Strain CSTR-G	EF 656617.1	99	H_2 fermentor
E	Unculture bacterium Clone HPR 146	DQ 482730.1	100	anaerobic sludge

Table 4-10 Information of sludge samples for PCR-DGGE

Lane no.	HRT (h)	Sampling time (day)
1	Seed sludge	0
2	24	10
3	12	20
4	8	30
5	4	40

4.11 The reactor performance with actual wastewater

As previously mentioned on the section 4-7, after the 4 h HRT test run ending, further feeding flow rate was gradually increased until reaching HRT of about 3 h, the sludge wash-out was begin to occurred and the accumulating biomass was noticed at the bottom of exit u-bend tube. The feeding pump was then stopped for awhile, after which the effluent deposited biomass was compiled and fed back to the reactor. After 3 days of operation, the reactor was sooner reached to steady-state stage at 4 h HRT by observation of the stable biogas produced. During which, the actual wastewater of spent malt extracted was continuously fed, replacing glucose substrate as the same concentration of about 7000 mg/l as COD, to the reactor operating for 7 consecutive days. The experimental results during steady-state condition are presented in Table 4-11 , also indicates the high efficiency of H_2 conversion and the same pattern of VFAs distribution,. At steady-state, the H_2 production rate were obtained in the average value of 14.90 ml/gVSS.h and 2.06 l/l.d, respectively, while the H_2 production and its content were 4737.65 ml/d at STP and 44.95%, respectively. These results are slightly lower as compared to those obtained from the previous study when using glucose as the feeding substrate. Possibly, these might be the decrease in the H_2 producing bacterial cell loss during the wash-out effect, evidenced by an initial biomass concentration left only 4096 g VSS/l. Therefore the stable H_2 producing reactor with mesophilic mixed anaerobic cultures operated with brewery waste yeast addition illustrated that biohydrogen production from spent malt extracted wastewater has a very high potential and feasible.

Table 4-11 Reactor performance with actual wastewater at HRT 4 hours

Day	CODinf (mg/l)	H_2 production (ml/d)	$\%H_2$	SHPR (ml/gVSS.h)	VHPR (l/l.d)	Acetate (mg/l)	Butyrate (mg/l)	Propionate (mg/l)
1	7280	5334.34	51.07	23.59	2.32	1216.38	986.94	961.34
2	7560	4157.69	39.04	18.39	1.81	1819.48	1235.07	923.76
3	7819	5866.93	54.00	25.95	2.55	1685.16	1152.72	890.67
4	7080	5334.34	34.00	15.44	1.52	1957.82	1419.13	841.39
5	7452	4157.69	50.21	23.31	2.29	1871.09	843.09	833.71
6	7020	5866.93	43.88	20.51	2.02	1374.78	827.71	536.93
7	7360	5334.34	42.14	19.49	1.92	1213.56	845.87	669.13
Avg	7367.28 ± 255.86	4737.65 ± 748.27	44.95± 6.66	14.90 ± 2.35	2.06 ± 0.33	1591.18 ± 293.36	1044.36 ± 213.16	808.13 ± 140.41

Mean ± S.D.

4.12 Confirmation Test for Continuous Operation

According to the previous results obtained from the continuous operation (Table 4-6 and Table 4-9), demonstrated an effect of hydraulic selection on the microbial population. It is clearly shown that at a high volumetric dilution rate (HRT of 4 h), the efficient hydrogen producer still remained in the reactor, particularly *C. pasteurianum*. As well known, this specie is of an important role in taking account for a mass production of H_2 gas. To ensure the experimental result from HRT shifting effect, the continuous feeding investigation was run again in an increment of HRT of 4 to 24 h, by running backward from 4 h to 24 h. The experimental data was shown in Table D-4, APPENDIX D.

From previous experience, the heat-treated sludge used in this study was an active biomass, because of its well-acclimation process (as described in detail in item 4.6). Thereby a dynamic increase in biogas production was observed once the substrate fed to the reactor which was initially set at HRT of 12 h for the process start-up. After glucose conversion rate reached to 95% within a couple of day, the reactor HRT was shortened to 4 h operation. Steady gas production was prior observed on day 5 afterward. After steady-state condition was reached, HRT was varied between 4 to 24 h for five runs. The five steady-state conditions, marked as Runs 1-5, were at least maintained for 2 weeks at each HRT before changing to the new one. The results obtained at steady-state conditions are summarized in Table 4-12, showing similar manner of H_2 production of which obtained from the previous study.

The biogas produced contained of H_2 and CO_2 and none of methane gas was found throughout this study. Hydrogen gas accounted for about 40 to 60 % of total biogas occurred in all Runs. As summarized in Table 4-12, H_2 yield of 2.70 mol/mol glucose consumed was obtained in Run 1, at which the reactor operated at HRT 4 h. H_2 yield decreased to 1.91 mol/mol glucose consumed when HRT was increased to 8 h in Run 2. This value was in close

to the result obtained by Zhang *et al.*, 2006 based on the study carried out treating glucose in CSTR operated at HRT 8 to 12h. When the reactor was shifted back to 4 h HRT operation, H_2 yield was obtained as 2.81 mol/mol glucose and varied between ± 0.08 mol/mol glucose during steady-state stage on day 35 until day 42.

Table 4-12 Reactor performance at steady-state (mean±95 % confidence interval)

Run No.	Operating time (days)	HRT (h)	VSS (g/l)	Glucose conversion rate(%)	H_2 yield (mol H_2/ mol glucose)	SHPR* (ml/gVSS.h)	VHPR** (L/L.d)	% H_2
1	3-14	4	11.08±0.02	22.73±1.65	2.70± 0.08[a]	19.45± 1.08	5.17±0.29	56.73±3.29
2	15-26	8	8.86±0.02	53.88± 1.52	1.90± 0.22[b]	20.27± 2.14	4.31±0.46	57.68±6.57
3	27-42	4	6.65±0.02	23.40± 0.93	2.81± 0.08[c]	34.76± 1.53	5.53±0.24	60.78±2.47
4	43-57	12	6.05±0.01	90.36± 0.64	0.69± 0.10[d]	12.03± 1.78	1.74±0.26	40.33±6.45
5	58-72	24	5.84±0.01	100± 0.0	0.30± 0.04[e]	4.10± 0.27	0.59±0.05	22.99±1.52

a) n=6 b) n=5 c) n=8 d) n=6 e) n=6

*SHPR = Specific hydrogen production rate

**VHPR = Volumetric hydrogen production rate

The H_2 yield of 0.69 and 0.30 mol/mol glucose were obtained as HRTs were increased to 12h to 24 h in Run 4 and Run 5, respectively. The other H_2 productivity results such as VHPR and SHPR were consistent to those results obtained in the previous study at each HRTs, whereby glucose conversion rate increased in correlation with the increased HRTs from 4 to 24 h. However, it is interesting to note that efficiency of glucose conversion rate of which the reactor operated at 8 h (Run 2), were ca. 53.88 %, lower than the result (84.94 %) obtained at the same HRT from the previous study (Table 4-6), with the exception of HRT 4 h, 12h and 24 h which obtained as nearly the same value. This may likely to be the mechanism of hydraulic selection at 4 hHRT at the beginning of the first Run

that caused to change in the microbial structure resulting in alteration of glucose utilization. Because H_2 yield could be calculated by dividing volume of H_2 gas produced by quantity of glucose utilized by microorganism. Therefore, as the quantity of glucose consumed was reduced (smaller divider) whereas the amount of comparable H_2 production was too closed to those generated from the reactor operated at the same HRTs, consequently, the increased of H_2 yield was obtained .

Table 4-13 Average results of VFA obtained from reactor operated at HRT of 4h (steady-state duration)

Run No.	Acetate (mg/l)	Butyrate (mg/l)	Acetate (mM)	Butyrate (mM)	A/B ratio	H_2 yield (mol/mol glucose)
4/1	2034	1805	33.91	20.51	1.65	2.87
1/2	2292	2455	38.2	27.90	1.37	2.70
3/2	2117	2368	35.29	26.91	1.31	2.81

The biomass concentration (VSS) in the reactor was in the range of 5.84 to 11.08 g/l during the 72 consecutive days of operation. The effluent SS measured was always kept low in all runs (see Table D-4), except for the first start up time regardless of whether high or low hydraulic flow rate, which leading to the reactor SRT could be done over the desirable value for high-rate anaerobic process (~20 days) as recommended by Metcalf and Eddy (2004), suggesting an effective result of the modified CSTR used in this study.

The level of volatile metabolic products in the reactor effluent present in Table D-4 (APPENDIX D) showed that acetate and butyrate were dominant soluble products, with no ethanol was measured in all runs. The distribution of VFAs observed in this study was similar

to those obtained from the previous work. However, it should be noted that at the highest H_2 yield of 2.70 ±0.08 and 2.81±0.08 mol H_2/mol glucose consumed were obtained in Run 1 and Run 3, respectively, the average molar ratio of acetate : butyrate(A/B) was obtained as more than1.0 (Table 4-13).The same ratio of A/B and highest yield of H_2 were also obtained from the reactor operated at the same retention time in the previous study (Table 4-8). This implied that the high yield of H_2 were consistent with a high molar acetate : butyrate ratio as more H_2 is produced with acetate than with butyrate.

4.13 Discussion

The comparable hydrogen yield of 2.70-2.87 mol H_2/mol glucose at 4 h HRT (see Table 4-13), represent ~70 % of the theoretical yield. Such a yield was higher than those of other studies reported in the literature (1.1-2.6 mol/mol glucose) (Table 4-7). But these yields are closed to that yield reported by Ginkel and Logan (2005). They found that the decreasing of glucose loading rate strategy from 18.9 to 0.5 g/h could increase H_2 yield from 1.7 to 2.8 mol H_2/mol glucose as resulting in less inhibition of accumulating H_2 gas occurred.

In Clostridia system, H_2 could be produced from glucose and get along with acetate and butyrate according to (Thauer *et al.*, 1977)

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + \mathbf{4H_2}\ \mathbf{(4\ ATP)} \quad (1)$$

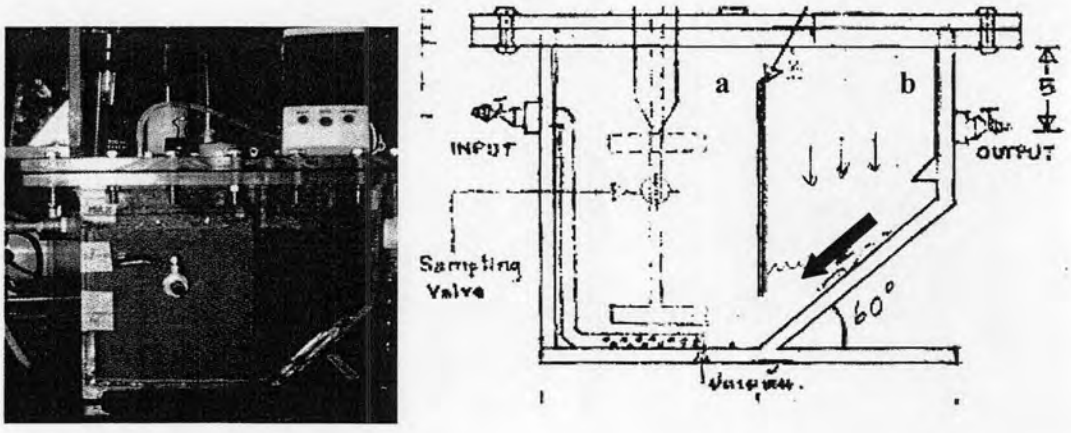
$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + \mathbf{2H_2}\ \mathbf{(3\ ATP)} \quad (2)$$

Stoichiometrically, each mol of glucose produces a maximum of 4 mol hydrogen assuming acetate being as the sole by-product, and also resulting in a net production of 4 mol of ATP. The main limitation for producing of the highest yields of H_2 is the inhibitory effect

of H_2 partial pressure (Angenent *et al.*, 2005), which may disturb the transfer of electron from glucolysis through reducing equivalents such as NADH or Fd_{red} (as already explained in greater detail in item 2.3.1, Chapter 2). In order to keep for the maximum ATP production, some bacteria (For example, *C. pasteurianum*) divert electron in NADH to the production of butyrate resulting in a decrease in H_2 yield and the production of 3mol of ATP (Equation (1) and (2))(Ginkle and Logan, 2005). In addition, Crabbenbaum *et al.*, (1985) found that the average ATP yield was 3.27±0.02 mol ATP / mol glucose, which corresponds to H_2 yield of 2.7 mol H_2/mol glucose. This value for H_2 yield is closed to the results obtained here in this study.

Moreover, this high H_2 yield obtained from this study may be a result from the balanced energy in H_2 bacterial cell as aforementioned. It was thought that the increase in yield of H_2 should be dealing with the reactor configuration used in this study as well. Namely, in continuous flow system, it is possible to maintain a constant high-rate of microbial growth by controlling the operation parameters such as HRT/organic loading, solids retention time (SRT) (Brosseau and zajic, 1982) and etc.

The results obtained in this work indicate that decrease in HRT can enhance H_2 production rate. As shown in Table 4-12, the maximum H_2 production rate was obtained by a shift of HRT to 4 h, corresponded to dilution rate (D value) of 0.25 h^{-1}. Although shorter HRT seem to lead to larger H_2 production rate, the D value should not be allowed to exceed the critical value (μ_{max}) of H_2 producing bacteria (0.172 h^{-1}-0.333 h^{-1}) (Table 2-6), to avoid reactor failure due to wash-out of H_2 producing bacteria (Chen *et al.*, 2001). The ordinary CSTR reactor used for studying H_2 production which allowed HRT and SRT to be varied dependently, always encounter the sludge wash-out problem when the HRT is shortened to 4h (D ~.μ_{max}). But the reactor used in this study was CSTR+RS (Figure 4-23) by using the same concept as activated sludge process. The effective results are :-



A B

Figure 4-23 A is photograph showing actual CSTR+RS during operated at steady-state of HRT of 4 h , B is front view of the model
a = reactor compartment, b = sedimentation zone
Angle 60° used for self sludge clearing in sedimentation zone

1. The reactor HRT and SRT are varied independently, and allows the effluent sludge recycle back automatically which can keep higher biomass to the reactor compartment all the time.
2. Size & weight of flocs will increase, that is easy to settle and more efficiency in SRT controlling
3. There will be young & active sludge occurred which active cell could stay in a log growth phase that would overcome poor H_2 producing biomass resulted from short HRT operation or at dilution rates come closer to its critical value (D ~.μ_{max}).

Therefore, besides the reactor operation at low HRT (high hydraulic flow rate) and maintaining high biomass in the reactor are preferable to enhance H_2 production in

continuous culture, the appropriate reactor as proposed in this study is also contributed to reach the maximum H_2 gas production. This finding, the modified CSTR+RS is able to be used as a tool to enhance H_2 yield and should be applied in design of a high-rate biohydrogen reactor in large scale.