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Enhanced biohydrogen production from beverage wastewater: process performances during various hydraulic retention time and their microbial insights

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Abstract

This study demonstrates the feasibility of the continuous hydrogen production from beverage industrial wastewater (BW) in a continuously-stirred tank reactor (CSTR) using enriched mixed microflora (EMC) at mesophilic condition. Various hydraulic retention time (HRT) (ranging from 6 to 1.5 h with an influent substrate concentration of 20 g/L $_{\text{hexose-equivalent}}$) have been evaluated to find out peak hydrogen production rate (HPR) and operational stability of the bioreactor. The results showed that peak HPR of 37.5 L H_2/L-d was observed at HRT 1.5 h , contrastingly, the maximum hydrogen yield (HY) of 1.62 mol H_2/mol hexose attained at HRT 6 h. This HPR value is quite higher than other organic wastewaters reported. Major soluble metabolic products formed were butyric, lactic and acetic acids. Microbial community composition characterized by PCR-DGGE analysis revealed that *Clostridium* sp. was the dominant one. HRT-dependent trends influenced the HPR and HY. Peak energy production rate of 441 KJ/L-d was achieved at lower HRT (1.5 h) evaluted.

Keywords: *Bio-hydrogen, Beverage wastewater, Butyrate, HRT, PCR-DGGE*

1. INTRODUCTION

The ever increase in oil prices, scarcity of fossil fuel reserves, global warming and climate changes are the major driving forces for searching environmental friendly and renewable energy carriers. In recent years, various biofuels (bioethanol, biobutanol, and biodiesel) are proposed to reduce fossil-fuels consumption and carbon foot prints[1]. Among them, hydrogen seems to be promising than other fuels because it produces only water after combustion, carbon neutral and satisfies the environmental benefits of practicing as a fuel for a daytoday life Moreover, hydrogen can be produced biologically using anaerobic microorganisms from renewable organic waste materials like industrial wastewaters, lignocellulosic biomass and food wastes [2-4].

In earlier studies, regarding the biohydrogen production, only glucose and sucrose are widely studied substrates for continuous system, which has been realized now as non-economic process towards industrial scale applications. Therefore, exploitation of wastewaters is recommended for cost-effective and sustainable bioprocesses. The utilization of wastewaters for biohydrogen production have been increased in recent years and usually conducted in batch and continuous modes of operation [5]. Most investigations on continuous hydrogen production from industrial wastewaters of cheese whey, coffee drink-manufacturing, condensed soluble molasses, tofu processing, sugary wastewater and molasses [6-11] were conducted in continuous stirred-tank reactors (CSTR) due to better mass-transfer and mixing. Selection of cost-effective feed stock is an important crieterian to acieve the success in the large scale H_2 generation.

In an economical and industrial perspective, continuous operation is prefeered, since it could save time and other capital cost, especially, while using organice wastewater as feedstock. Previously, mixed cultures were employed for the hydrogen fermentation, however, their

stability was not feasible, since the major changes occur in the continuous operation due to the shifts in the microbial community. This could be solved by preparing the enriched mixed cultures (EMC) as proposed in some investigations [12, 13], since EMC provide stability in the operation and also comprises mostly the efficient hydrogen producers.

There are several strategies to improve bio-hydrogen productivity. Among them, evalauationg the importance of HRT during its reduction in the continuous system is an important factor which can regulate the metabolic flow of microorganisms and elimination of non-hydrogen producing bacteria at lower flow rates. Our recent finding narrated that BW could produced a stable HPR of 13 L/L-d at 8 h HRT [14]. Moreover, many other reports showed that short HRTs (like 4-0.5 h) also showed significant effect on efficient hydrogen production performances [7, 9, 15, 16].

Thus, to seek the influence of HRT for further improving the HPR, this study was aimed to find out the effect of HRTs (6 to 1.5 h) on continuous hydrogen production from BW using selectively enriched mixed culture (EMC) as seeding source. Additionally, the corresponding changes in microbial populations were accessed via PCR-DGGE sequencing to elucidate the microbial niche. The information obtained here is expected to be useful in developing future sustainable technologies for hydrogen production from cost-effective substrate (BW, as mentioned here) .[2]

2. Materials and methods

2.1 Microbial source and wastewater composition

The acclimatized enriched mixed culture from a CSTR hydrogen production bioreactor (pH 5.5, volatile suspended solids 3.23 g/L) fed with BW at 8 h HRT [14] was used as an inoculum

source in this study. The characteristics of the beverage wastewater (BW) were pH 2.6-3.4, chemical oxygen demand (COD) 760-900 g/L and total reducing-sugar 660-750 $g_{(hexose\ equivalent)}$/L. From this raw wastewater feedstock, 20 g total reducing-sugar $_{(hexose\ equivalent)}$/L substrate solution was made for the experiments.

2.2 Experimental setup for continuous operation

The schematic representation of the CSTR is shown in Fig. 1. A total volume of 2.5 L CSTR bioreactor with an effective working volume of 2.0 L was used. The start-up of the reactor was followed as mentioned in our previous study [14]. The reactor was maintained at a constant temperature of 37 °C without pH control. A basal endo medium [17] was used with the following ingredients (g/L): 5.24, NH_4HCO_3; 6.72, $NaHCO_3$; 0.125, K_2HPO_4; 0.1, $MgCl_2{\cdot}6H_2O$; 0.015, $MnSO_4{\cdot}6H_2O$; 0.025, $FeSO_4{\cdot}7H_2O$; 0.005, $CuSO_4{\cdot}5H_2O$; 0.00012, $CoCl_2{\cdot}5H_2O$. The CSTR was operated for 157 days, with HRTs 6 h and 4 h were maintained for 30 days after the post-start up, followed by 3 h HRT for 29 days, 2 h HRT for 45 days and 1.5 h for 23 days, respectively.

2.3 Analytical methods

Biogas composition (H_2 and CO_2) was analyzed with gas chromatography having a thermal conductivity detector (China Chromatograph 8700T). The biogas volume was measured using a wet-gas meter (Ritter, Bochum, Germany). The soluble metabolic products (volatile fatty acids and ethanol) concentration was detected by gas chromatography (Shimadzu GC-14A) using a flame ionization detector (FID). The COD, pH, and volatile suspended solids (VSS) concentrations were measured according to the procedures described in Standard Methods [18].

Dinitrosalicylic acid method was used to measure the total reducing-sugar concentration [19]. Hydrogen yield was computed as mol-H_2 mol^{-1} hexose.

2.4 Microbial community analysis

Microbial changes during different HRT operational conditions were assesed by denaturing gradient gel electrophoresis (DGGE) technique. Total genomic DNA collected at HRT 6h (day 24), 4 h (day 59), 3 h (day 85), 2 h (day 130), and 1.5 h (day 155) were extracted by using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene, Taiwan) following the manufacturer's instructions. The V6 regions of the bacterial 16S rRNA genes were subjected to Polymerase chain reaction (PCR) amplification and DGGE analysis as per the method described elsewhere [20].

2.5 Energy production analysis

The energy production rate (EPR) of biohydrogen and ethanol (*EPR*, kJ/L-d) was calculated as:

H_2 = HPR $\times HV_{H2}$ (1)

Where HPR was hydrogen production rate (mmol H_2/L-d), and HV_{H2} was heating value of hydrogen (286 J/mmol).

EtOH = Ethanol production rate $\times$ HV_{EtOH} (2)

Where Ethanol production rate (mmol ethanol/L-d), and HV_{EtOH} was heating value of ethanol (1366 J/mmol).

Total energy production rate (TEPR, KJ/L-d) = EPR $_{H2}$ + EPR $_{EtOH}$ (3) [21].

3. RESULTS AND DISCUSSION

3.1 Process performances under various HRTs

Five HRTs (6, 4, 3, 2 and 1.5 h) were operated in order to investigate the fermentative hydrogen production performances of BW. Figure 2 shows the profiles of biogas production rate (BPR), HPR, hydrogen yield (HY), pH, H_2 (%) and CO_2 (%) at various operational HRT conditions. The performance of the reactor under steady-state conditions of each HRT was summarized in Table 1. The organic loading rate (OLR) was increased from 80 to 320 g/L-d $_{\text{hexose equivalent}}$ by shortening the HRT from 6 h to 1.5 h. The reactor was started-up with a batch operation mode for 24 h and then switched to a continuous mode at 6 h HRT. After the steady-state condition (the condition reaching stable hydrogen production with less than 10% deviation) was obtained, HRT was gradually decrease to other designed values of 4 h, 3 h, 2 h and 1.5 h with each HRT having their own steady-state conditions. As seen from (Fig. 2), hydrogen content in the biogas mixture ranged from 39 to 45 %, which value is similar to the reports using condensed molasses solubles and molasses [10, 15]. The oxidation reduction potential (ORP) (-mV) was observed in the range of -343 to -453 mV, which is close to the optimal value for hydrogen production as indicated [22]. Furthermore, the pH range of 5.6 to 6.8 is favorable for metabolites and hydrogen production [23]. In our study, during HRT changes pH values were lied in the range of 5.7 to 6.4, which favors stable and efficient hydrogen production from BW.

HRT had impacts on hydrogen production rate (HPR) with shortening HRT (6 h to 1.5 h) gradually and increasing HPR (17.9 to 37.5 L/L-d). This result was similar to various reports on continuous hydrogen production and showed that lower HRT favoring higher hydrogen production rates due to increased OLR [5]. The maximum HPR (37.5 L/L-d at 320 g/L-d $_{\text{hexose equivalent}}$, HRT 1.5 h) obtained in this work was quite higher than a reported value of 9.8 L/L-d at HRT 3 h with similar OLR of 320 g COD/L-d using condensed molasses soluble substrate [24]. In fact, in the continuous mode the HPR was OLR dependent; increasing OLR from 80 g/L-d $_{\text{hexose}}$

equivalent to 320 g/L-d hexose equivalent favored hydrogen production rate but HY was significantly affected. Peak HY of 1.62 mol/mol hexose was achieved at low OLR (80 g L/L-d hexose equivalent, 6 h HRT), while lower HY of 1.05 mol/mol hexose was observed at higher OLR (320 g L/L-d hexose equivalent, 1.5 h HRT). The result of high HPR but low HY might relate to the changes in hydrogen-producing microbial community structure [24]. Table 2 shows the optimal operation conditions, maximal HPR and HY for various wastewater feedstocks used in continuous hydrogen production. As seen from Table 2, the HPR (37.5 L/L-d) obtained in this study was quite higher than other reported values using wastewater feedstock in continuous operation. The observed difference was mainly influenced by variations in the types of inoculum, substrate and other operational conditions (pH, OLR, HRT and temperature).

3.2 Metabolic end products distribution

In acidogenic hydrogen fermentation, HRT affects the production and distribution of soluble metabolic products (SMP). As shown in Table 1, butyrate and acetate were the major metabolic products, followed by lactate, ethanol, propionate and butanol. The SMP production varied significantly (in the range of 16.2 to 19.3 g COD/L) as HRT was decreased from 6 h to 1.5 h with lower SMPs production (16.2 g COD/L) and biomass concentration (2.73 gVSS/L) at shorter HRT of 1.5 h. Chen et al. [25] also observed the similar phenomenon of decreased SMP production and biomass concentration at lower HRTs using sucrose feedstock in a CSTR with HPR being affected at low HRT.

Volatile fatty acids distribution and their concentrations were HRT-dependent. Butyrate (HBu) accounted for 39.5% to 56.7% of the total SMPs indicating a butyrate-type fermentative pathway Fig. 3. Butyrate concentrations were 9.89 to 10.50 g COD/L at HRT 6-4 h and then

markedly decreased to 6.48 g COD/L with reducing HRT from 4 h to 1.5 h. Propionate concentration was observed to increase from 0.65 g COD/L to 0.89 g COD/L at decreasing HRT from 6 h to 4 h but to decrease to 0.43 g COD/L at HRT 1.5 h. However, acetate (HAc) concentration decreased gradually from 4.89 g COD/L to 2.26 g COD/L when HRT was reduced from 6 h to 1.5 h. Noted that lactate concentration rose dramatically from 0.24 g COD/L to 5.15 g COD/L, accounted for 1.3% to 31.8% of total SMP. The solvents ethanol and butanol accounted for 8% to 10.3% and 1.4 % to 4.2 %, respectively, with a negligible amount of formate (< 0.4%).

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2\,CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad (4)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2 \quad (5)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+ \quad (6)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+ \quad (7)$$

$$CH_3CHOHCOO^- + 0.4CH_3COO^- + 0.7\,H^+ \rightarrow 0.7CH_3CH_2CH_2COO^- + 0.6H_2 + CO_2 + 0.4H_2O \quad (8)$$

The distribution pattern of SMP as a function of HRT was dependent on OLR and microbial community activity [26, 27]. The high HBu/SMP and HAc/SMP ratios and lower reduced end products/SMP ratio, observed in this study indicates an efficient hydrogen generation system.

Moreover, butyrate and acetate production (Eqs 4 and 5), positively correlated to higher hydrogen production and their conversion ratio have been used to assess the performances of hydrogen production [28, 29]. As mentioned in Table 1, the HY decreased (1.62 to 1.05 mol/mol hexose) as the HBu/HAc ratio increased (0.80 to 1.14), in the HRT range of 6 to 1.5 h, indicating butyrate-mediated fermentative pathway observed under low HRTs which significantly affects the HY. This HBu/HAc ratio value was consistent with the values of previous findings and demonstrated lower concentration ratio of butyrate to acetate is associated with higher HY [30, 31].

Previous studies also indicated that increased OLR significantly affected the hydrogen production and distribution pattern of lactate and ethanol (Eqs 6 and 7) [32, 33]. Besides, coupled acetate and lactate pathways also exist (Eqn. 8) with the formation of hydrogen, CO_2 and butyrate [32]. The increased lactate concentration of 5.15 g COD/L at 1.5 h HRT significantly affected HY with low value of 1.05 mol/mol hexose, whereas higher yield of 1.62 mol/mol hexose was achieved at 6 h HRT with low lactate concentration of 0.24 g COD/L. The observed difference was attributed to that short HRT did not allow enough time for converting lactate to butyrate and hydrogen [32]. Ethanol concentration did not vary significantly (in the range of 1.35 to 1.99 g COD/L) at the tested HRTs (6 to 1.5 h). These concentration values are at the same level to some reported values [7, 15] using condensed molasses soluble in continuous hydrogen fermentation. Moreover, the production of lactate and ethanol under higher OLR (low HRT) was consistent with the findings of previous studies [34, 35].

The COD mass balances at various HRTs under steady-state conditions were computed based on the distribution of soluble metabolites, microbial biomass, and hydrogen (Table 3). The closure of COD balances of 87% to 99% indicates the accuracy of the experimental data. This proved that the reactor performance was reliable and the results are significant. In addition to that,

this confirms that the measurements and analysis of the gaseous and liquid products were accurate. The observed limited variation (less than 13%) in the COD recovery could be due to the marginal error of the determination methods used [36] .

3.3 Microbial community variation at various HRTs

Figure 4 shows the profile of the bacterial communities response under steady-state conditions of different HRTs (6 to 1.5 h) determined by using universal and clostridium-specific bacterial primer sets. As shown in Fig. 4, DGGE profiles were significantly different. It can be pointed out that changes in microbial community structure were attributed to the changes in HRT operation or in other words caused population shift in the mixed culture due to the wash-out of bacterial cells under higher dilution rate (i.e., shorter HRT). In DGGE band pattern analysis, each distinct band represented a specific species in the microbial population [37]. The excised selected bands were subjected to DNA sequencing analysis and their results were listed in Table 4. The homology strains were depicted in Figs 5 and 6. Figure 5 shows the genetic distance of the bacterial species obtained from BW at different applied HRTs. There are six distinct divisions among the *Clostridium* species, whereas *Klebsiella* Sp. and *Ruminococcus* Sp. possess a single group within in the total microbial populations. Figure 6 depicts the genetic distance of the *Clostridial* species based on the *Clostridium* specific primer sets. Among the *Clostridium* species, *Clostridium butyricum* possess the superior dominance over the other species, which is coincided with the butyrate-mediated fermenative metabolism of BW.

The bacterial communities prevailing at different HRTs were mainly composed of seven groups of bacteria namely *Ruminococcus albus*, *Clostridium butyricum, C. tyrobutyricum, C. pasteurianum, C. acetobutylicum*, *C. perfringenes* and *Klebsiella oxytoca.* At HRT 1.5 h with

peak HPR 37.5 L/L-d, the four species (*C. butyricum, C. tyrobutyricum, C. perfringenes* and *K. oxytoca*) were observed. *R. albus*, *C. pasteurianum and C. acetobutylicum* did not appear at HRT 1.5 h. Basically, *Clostridium sp.* are useful microorganisms in dark fermentative hydrogen production. Nevertheless, *Clostridium* and *Klebsiella* strains were dominant hydrogen producers observed at low HRT conditions, which serve as the efficient hydrogen production due to the wash-out of other non-competitive bacteria under higher dilution rates [38]. *Klebsiella* spp. has been reported as a potential facultative anaerobic hydrogen producer and were detected in continuous hydrogen production bioreactors fed with glucose, soft-drink wastewater or sucrose [37, 39, 40]. Moreover, *Klebsiella* spp. in a reactor consumes O_2 and assists to maintain a suitable anaerobic environment which might favor the growth of O_2-sensitive *Clostridium* sp and then result in efficient hydrogen production.

The shift in microbial populations (Fig. 4) at various HRTs showed a significant influence on soluble metabolites distribution as well as hydrogen production performances. *Ruminococcus albus* was observed at HRT 3 h but further lower HRT resulted in its disappearence. *C. pasteuarnium* and *C.acetobutylicum* also disappeared at low HRT of 1.5 h. The lower HY 1.05 mol/mol hexose at HRT 1.5 h probably due to the wash-out of these populations under low HRT. Besides, C. *butyricum*, *C. tyrobutryicum* and *C. perfringenes* were the predominant populations observed at all tested HRTs. The Clostridial species (*C. butyricum, C. tyrobutyricum, C. perfringenes*) detected at HRT 1.5 h have been reported as potential hydrogen-producing bacteria in continuous operations [15, 32, 38, 41]. In general, Clostridial spp. exhibits butyrate-type hydrogen fermentation with formation of acetate, lactate and ethanol[42]. *Klebsiella* spp. exhibits mixed acid type hydrogen fermentation with cogeneration of ethanol and acetate. This results agreed with the SMP analysis and imply that the dominant metabolites formed during BW fermentation were

butyrate, lactate, acetate and ethanol. The optimal operation conditions (low HRT of 1.5 h and high OLR of 320 g/L-d $_{\text{hexose equivalent}}$) observed in this study favored the growth of efficient hydrogen producing bacteria and resulted in enhanced hydrogen production. The DGGE analysis clearly showed that HRT significantly affected the composition of microbial community structure in continuous hydrogen production.

3.4 Energy production rates

The energy production rates (EPR) at various HRTs were calculated based on the higher heating combustion value of hydrogen and ethanol. As shown in Table 5, the EPR values increased as HRT dropped from 6 to 1.5 h. In other words, it was also due to the increased OLR (Table 5). Peak EPR (441 KJ/L-d) was obtained at 1.5 h HRT, while minimum EPR (222 KJ/L-d) was observed at 6 h HRT. This is similar to a report by Han et al.[31] indicating that increased OLR improved energy production rate. Ethanol production rate dropped at 1.5 h HRT, whereas hydrogen production was not significantly affected at low HRT of 1.5 h; this could be due to the changes in microbial community structure. The EPR obtained in this study was lower than a value of 457 KJ/L-d [43] in an CSTR operation using sugar beet molasses. However, it was higher than a value of 113 KJ/L-d [44] using diluted sugar cane stillage wastewater. The EPR analysis showed that BW could be used as an efficient low-cost feedstock for sustainable bio-hydrogen and ethanol production.

3.5 Significance of the study

According to the experimental results, low HRT with high OLR resulted in efficient hydrogen production. Maximum HPR (37.5 L/L-d) was observed at lower HRT (1.5 h and OLR

320 g/L-d $_{\text{hexose equivalent}}$). Moreover, HRT significantly influenced the microbial community structure, biomass concentrations, soluble metabolic products distribution and hydrogen production performances in the CSTR bio-hydrogen fermentor. Therefore, using short HRT to have a high OLR is a functional strategy for efficient hydrogen production from a beverage wastewater.

Increased lactate production (0.24-5.15 g COD/L) significantly affected the hydrogen yield but not HPR; the reduction phenomenon of HY was due to the increased hydrogen partial pressure at high OLR. This high OLR diverted the metabolic flux towards lactate and ethanol and decreasing the activity of hydrogenase. As indicated by Kim et al.[45] CO_2 sparging could reduce the accumulation of lactate in a bioreactor, thus CO_2 gas sparging could be one of the useful strategy to improve the hydrogen production performance in a continuous operation.

The microbial community analysis demonstrated the dominance of efficient hydrogen-producing bacteria (*C. butyricum, C. tyrobutyricum, C. perfringenes* and *K. oxytoca*) at low HRT (1.5 h) which resulted in efficient hydrogen production from BW. However, low biomass concentration of 2.7 gVSS/L was observed at 1.5 h HRT. The cell biomass wash-out under low HRT is a common behavior in a CSTR bioeactor due to lack of granule forming ability/immobilized structure to retain the biomass. Thus, immobilized cells operation is further recommended to study and compare the performances of hydrogen fermentation from BW[46].

4. CONCLUSIONS

HRT affects the hydrogen production rate, hydrogen yield and liquid metabolite products of a biohydrogen production from beverage wastewater in different HRT-dependent trends. Short HRT of 1.5 h can result in peak hydrogen production rate and energy production rate. At HRT 1.5 h, peak HPR and energy production rate were 37.5 L/L-d and 441 KJ/L-d, respectively, with *C.*

butyricum, C. tyrobutyricum, C. perfringenes and *K. oxytoca* being the dominant microflora. HY peaked at 6 h HRT with a value of 1.62 mol/mol hexose. This study shows that BW can be used as an efficient low-cost feedstock for sustainable bio-hydrogen production.

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Table 1 Fermentation performance under steady-state conditions at different HRTs

Parameters	Operational conditions				
	I	II	III	IV	V
Steady-state days	21-30	51-60	82-89	109-134	143-154
Hydraulic retention time (h)	6	4	3	2	1.5
Organic loading rate (g substrate/L-d)	80	120	160	240	320
Volumetric hydrogen production rate (L/L-d)	17.92±0.19	21.65±0.39	26.59±0.34	32.85±0.55	37.56±0.75
Hydrogen yield (mol/mol hexose $_{utilized}$)	1.62±0.03	1.30±0.02	1.25±0.02	1.06±0.02	1.05±0.03
Substrate degradation rate (%)	97.9±0.99	97.41±0.43	93.47±0.73	92.0±0.95	78.33±1.26
Ethanol (g COD/L)	1.43±0.09	1.52±0.05	1.99±0.15	1.65±0.09	1.35±0.20
Butanol (g COD/L)	0.25±0.01	0.22±0.01	0.79±0.01	0.28±0.01	0.51±0.01
Propionate(g COD/L)	0.65±0.01	0.89±0.03	0.60±0.04	0.54±0.05	0.43±0.01
Acetate (g COD/L)	4.89±0.18	4.49±0.17	3.76±0.07	3.05±0.07	2.26±0.10
Butyrate (g COD/L)	9.89±0.71	10.50±0.25	9.47±0.29	8.23±0.59	6.48±0.22
Lactate (g COD/L)	0.24±0.10	1.44±0.15	2.74±0.15	3.49±0.14	5.15±0.24
Formate(g COD/L)	0.05±0.01	0.04±0.02	0.07±0.01	0.03±0.02	0.05±0.01
TVFA (g COD/L)	15.74±0.70	17.37±0.74	16.53±0.15	15.35±0.10	14.35±0.26
SMP (g COD/L)	17.43±0.63	19.10±0.62	19.33±0.25	17.28±0.19	16.22±0.40
VSS (g/L)	3.36±0.12	4.55±0.15	3.96±0.20	3.47±0.10	2.73±0.18
B/A ratio (%)	0.80±0.06	0.94±0.05	1.00±0.03	1.07±0.01	1.14±0.01

TVFA, total volatile fatty acid, = Propionate+ acetate+ butyrate+ lactate+ formate; SMP, soluble metabolite product, = Ethanol+ butanol+ TVFA; VSS, volatile suspended solid

Table 2 Comparison of hydrogen yield (HY) and production rates (HPRs) of various wastewaters in continuous hydrogen fermentation

Inoculum	Substrate	Reactor type	pH	OLR (g COD/L)	HRT (h)	Temp (°C)	HY (mol/mol substrate)	HPR (L/L-d)	References
AS	CMS	CSTR	5.5	40	3	35	0.9	9.86	[7]
ADS	TPWW	CSTR	5.5	20	8	60	1.20	8.17	[9]
ADS	TPWW	MBR	5.5	43.4	4	60	1.45	19.86	[9]
AS	Molasses	CSTR	4.4	8	5	35	N.A	7.47	[10]
AGS	CWWW	CSTR	5.9	138.6**	6	37	2.8	28.47	[11]
AS	CMS	CSTR	5.5	40	0.5	37	2.02mmol/ H_2/g COD	14.04	[15]
ADS	Molasses	EGSB	4.4	120	2	35	3.47	17.04	[16]
ADS	CSWW	Novel reactor	5.5	27	8	37	3.2	34	[47]
AS	Beet sugar wastewater	CSTR	4.5	18	8	35	N.A	10.8	[48]
AS	TPWW	CSTR	5.5	20	8	35	N.A	1.73	[49]
EMC	BW	CSTR	6.3	20**	1.5	37	1.05	37.5	This study

AS- anaerobic sluge; AGS- anaerobic granular sludge; ADS- anaerobic digester sludge; EMC- enriched mixed culture; CWWW- Cheese whey wastewater; CMS- condensed molasses soluble; TPWW- tofu processing wastewater; CSWW-corn syrup wastewater; BW-beverage wastewater; **-g/L; CSTR- continuously stirred tank reactor; MBR- membrane bioreactor; EGSB- expanded granular sludge bed reactor.

Table 3 COD mass balance in the hydrogen fermentation of immobilized cells bioreactor at various HRTs

HRT (h)	$COD_{sub,in}$ (g COD/h)[a]	$COD_{sub,res}$ (g COD/h)[b]	COD_{SMP} (g COD/h)[c]	COD_{H2} (g COD/h)[d]	COD_{Bio} (g COD/h)[e]	COD_{sum} (g COD/h)[f]	COD Balance (%)[g]
6	4.03	0.07	2.90	0.47	0.58	4.03	99.9
4	6.05	0.13	4.77	0.56	0.50	5.98	98.9
3	8.06	0.39	6.44	0.70	0.34	7.88	97.7
2	12.10	0.85	8.64	0.86	0.19	10.55	87.2
1.5	16.13	3.06	10.81	0.98	0.11	14.97	92.8

[a]$COD_{sub,in}$: g COD/h of influent substrate, calculated by (substrate concentration (mg COD/L)* feeding rate (L/h)).
[b]$COD_{sub,res}$: g COD/h of residual substrate in the effluent, calculated by (CODsub,in * (1 - substrate utilization)].
[c]COD_{SMP}: g COD/h of soluble microbial products (SMP), calculated by (SMP concentration (mg COD/L) *feeding rate (L/h)).
[d]COD_{Bio}: g COD/h of biomass in the effluent, calculated by (mg cell/L *feeding rate (L/h) * 1.42 mg COD/mg VSS/L), assuming that cell formula is $C_5H_7O_2N$ [50].
[e]COD_{H2}: g COD/h of H_2 evolved, calculated by (mol H_2/h * 16 g COD/g H_2).
[f]COD_{sum}: g COD/h, sum of residual substrate+ SMP+ biomass+ H_2.
[g]COD balance (%): [COD_{sum}]/ [$COD_{sub,in}$]*100

Table 4 Affiliation of band sequence (retrieved from DGGE gel) determined using BLAST algorithm

Primer sets	Band	Species	HRT				
			6	**4**	**3**	**2**	**1.5**
EUB 968f-GC & 1392r	**1**	*Ruminococcus albus* (accession no. NR_113032.1)	-	-	+	-	-
	2	*Clostridium butyricum* (accession no. NR_042144.1)	-	-	+	+	+
	3	*Clostridium tyrobutyricum* (accession no. NR_044718.2)	+	+	+	+	-
	4	*Clostridium butyricum* (accession no. NR_042144.1)	+	+	+	+	+
	5	*Clostridium pasteurianum* (accession no. NR_104822.1)	-	-	+	+	-
	6	*Clostridium acetobutylicum* (accession no. NR_074511.1)	+	+	-	+	-
	7	*Klebsiella oxytoca* (accession no. NR_102982.1)	+	-	+	+	+
	8	*Clostridium perfringens strain 13* (accession no. NR_074482.1)	+	+	+	-	-
Chis 150f GC & ClostIr	9	*Clostridium perfringens* (accession no. JF499889)	+	+	-	+	+
	10	*Clostridium tyrobutyricum* (accession no. NR_044718.2)	-	+	+	+	+
	11	*Clostridium butyricum* (accession no. NR_042144.1)	-	+	-	-	-
	12	*Clostridium butyricum* (accession no. NR_042144.1)	+	+	+	+	+

'+' = appearance; '- ' = non appearance

Table 5 Total energy production rate at various HRTs

HRT (h)	Production rates (mmol/L-d)		Energy production rate (KJ/L-d)		Total energy production rate (KJ/L-d)
	Hydrogen	Ethanol	Hydrogen	Ethanol	
6	533.93	14.5	201.84	20.35	222.20
4	705.75	14.9	243.79	21.65	265.44
3	852.40	15.85	300.80	28.39	329.18
2	1051.73	20.78	369.39	23.51	392.90
1.5	1291.57	17.21	422.35	19.17	441.52

Figure Captions

Figure 1. Schematic diagram of the CSTR used in the study. 1. Beverage wastewater tank; 2. Fermentor; 3. Input flow of wastewater; 4. pH and ORP monitors; 5. Magnetic stirrer; 6. Gas and liquid separator; 7. Liquid effluent; 8. Water seal; 9. Gas meter; 10. Input flow of buffer; 11. Buffer tank.

Figure 2 Performance of CSTR at various hydraulic retention times.

Figure 3 Soluble metabolic product distributions at various hydraulic retention times.

Figure 4 DGGE analyses of the reactor samples at various steady-state operational HRTs using primer sets (a) Universal Eubacterial primer set, and (b) *Clostridium*-specific primer set.

Figure 5: Phylogenetic tree showing the species relatedness to the sequences identified in the mixed cultures of universal eubacterial primer set. The tree based on maximum composite likelihood method was constructed using neighbor-joining algorithm with 1,000 bootstrapping. *E. coli* was selected as the outgroup species. The scale bar 0.02 represents substitutions per nucleotide position. Numbers at the nodes are the bootstrap values.

Figure 6: Phylogenetic tree of Clostridium-specific primer set. The tree based on maximum composite likelihood method was constructed using neighbor-joining algorithm with 1,000 bootstrapping. *E. coli* was selected as the outgroup species. The scale bar 0.02 represents substitutions per nucleotide position. Numbers at the nodes are the bootstrap values.

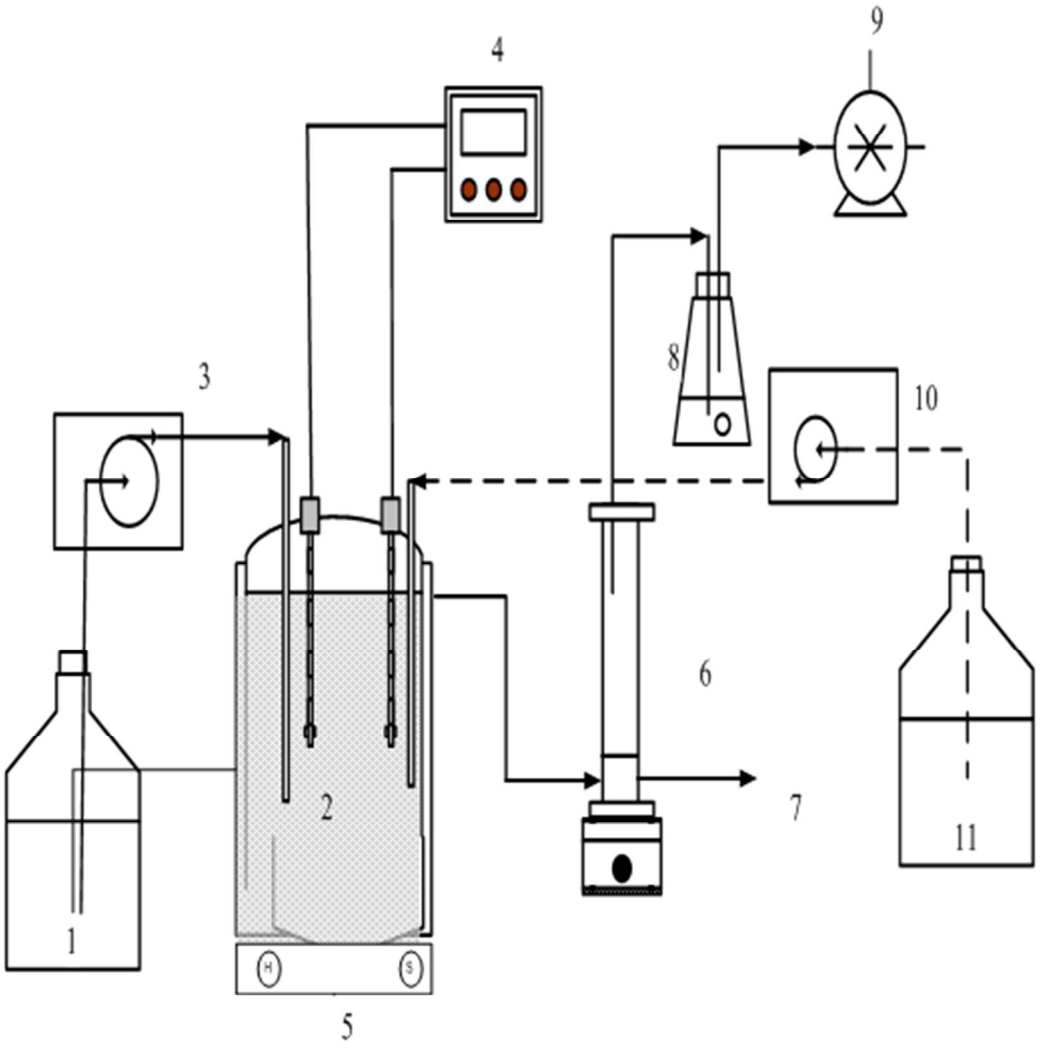


Figure .1

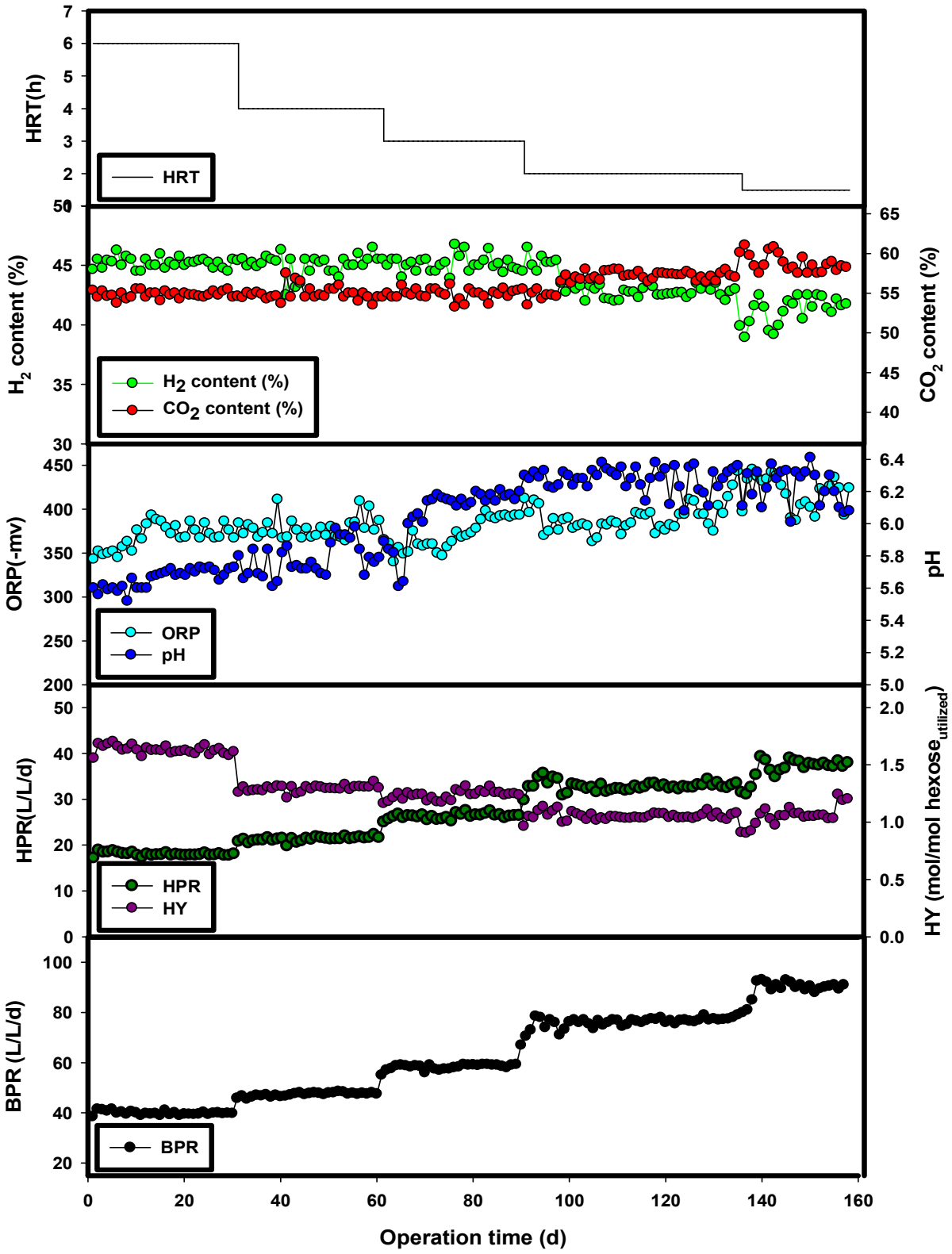


Figure.2

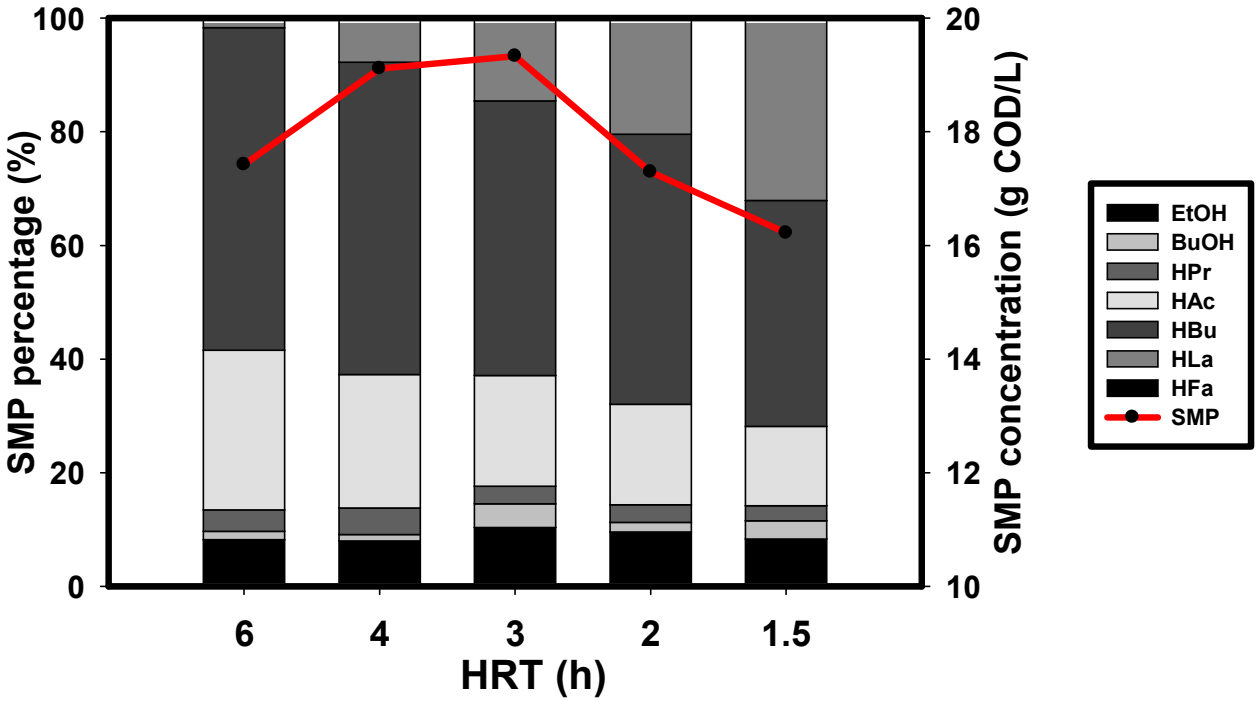
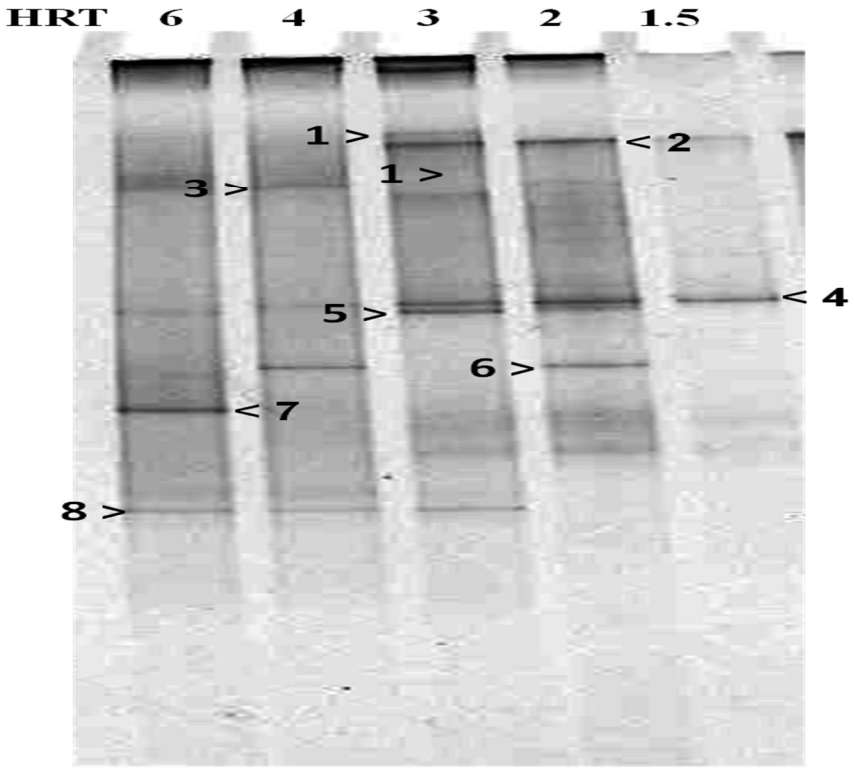
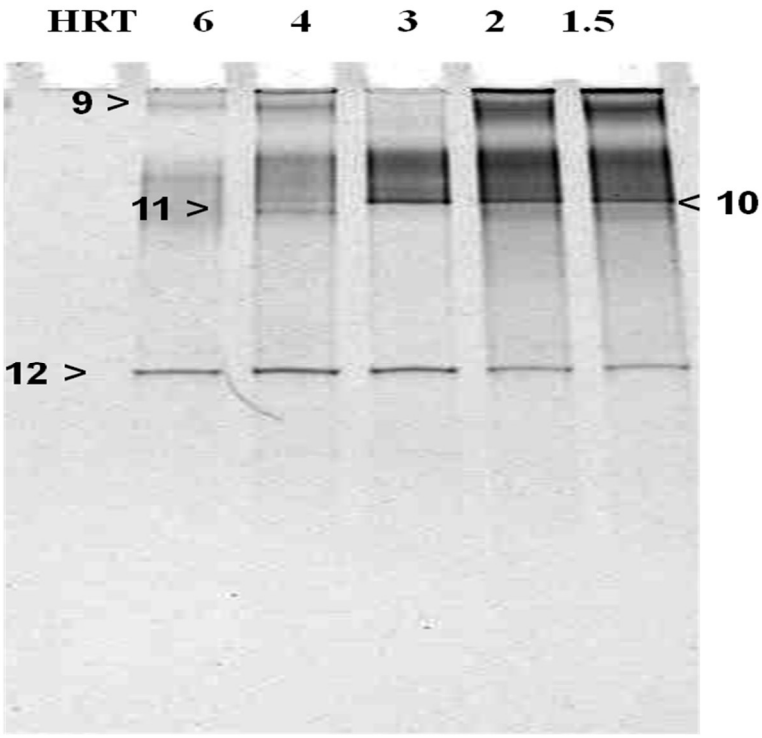


Figure.3



(a)



(b)

Figure.4

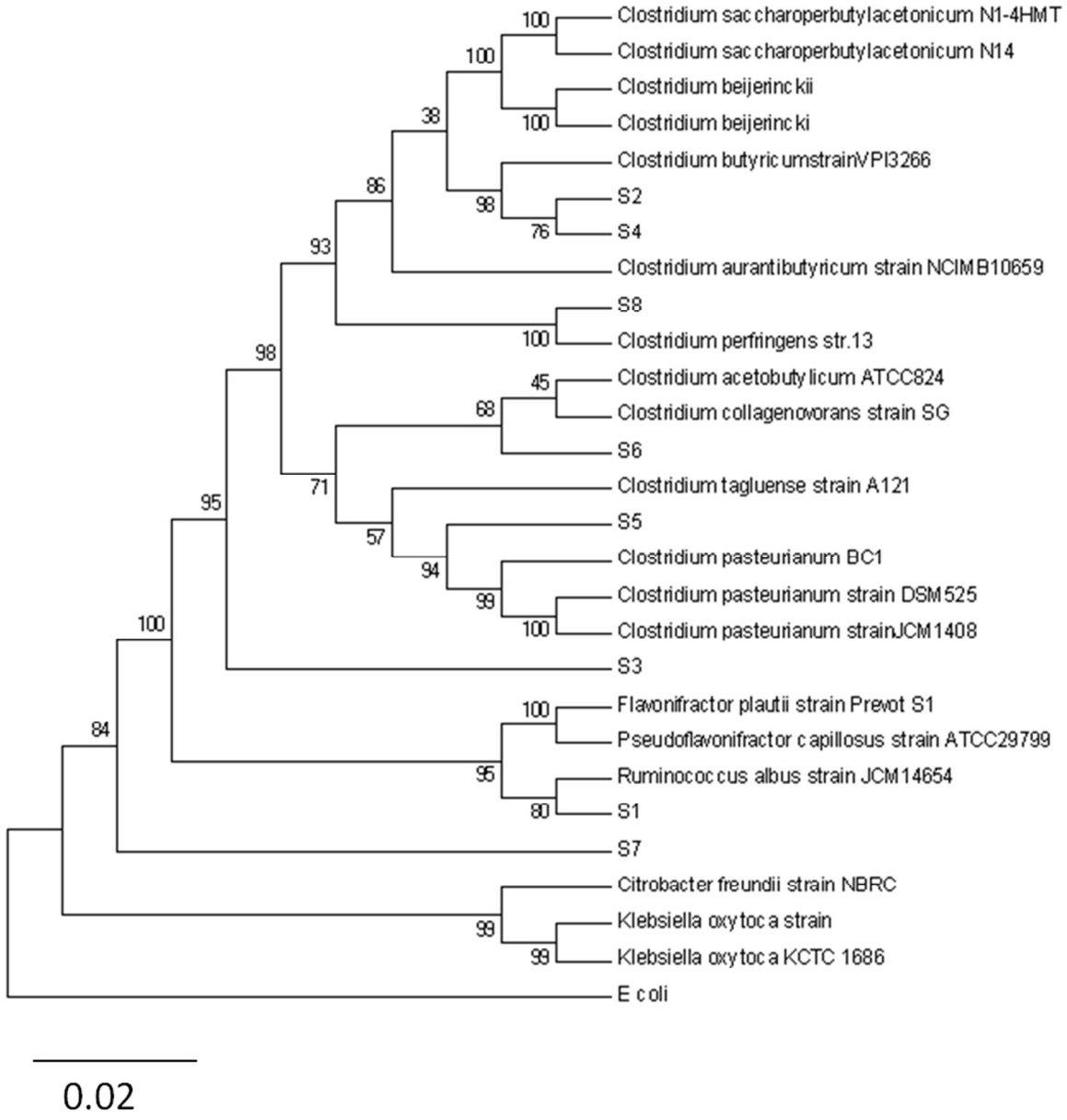


Figure .5

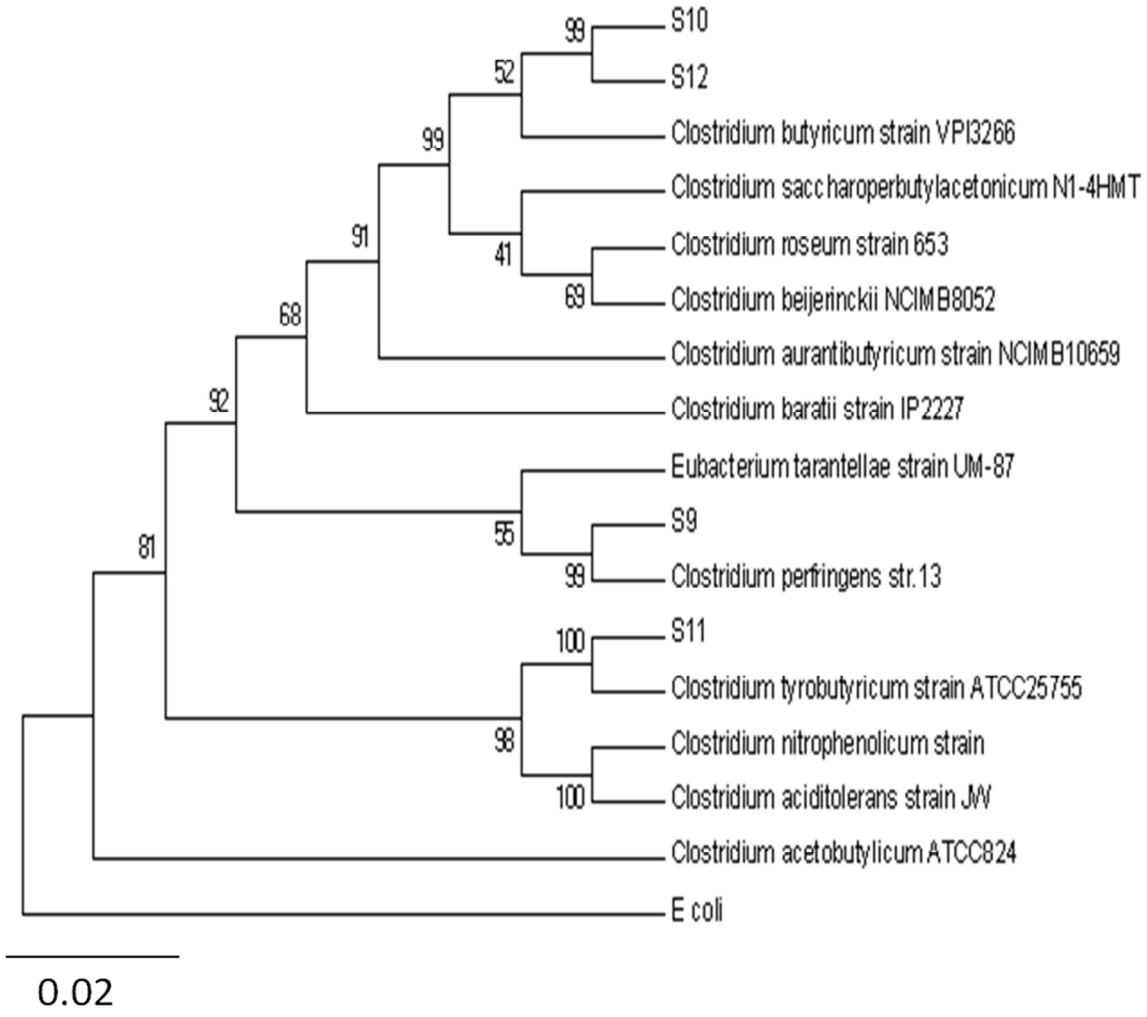


Figure.6