Biotechnology and Bioprocess Engineering 17: 133-144 (2012)
DOI 10.1007/s12257-011-0248-7

RESEARCH PAPER

Response Surface Methodology Analysis of Anaerobic Syntrophic Degradation of Volatile Fatty Acids in an Upflow Anaerobic Sludge Bed Reactor Inoculated with Enriched Cultures

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Received: 3 June 2011 / Revised: 11 September 2011 / Accepted: 16 September 2011

Abstract Anaerobic oxidation of volatile fatty acids (VFAs) as the key intermediates is restricted thermodynamically. Presently, enriched acetogenic and methanogenic cultures were used for syntrophic anaerobic digestion of VFAs in an upflow anaerobic sludge bed reactor fed with acetic, propionic, and butyric acids at maximum concentrations of 5.0, 3.0, and 4.0 g/L, respectively. Interactive effects of propionate, butyrate and acetate were analyzed. Hydraulic retention time (HRT) and acetate oxidizing syntrophs and methanogen (hydrogenotrophs) to syntrophic bacteria (propionate- and butyrate-oxidizing bacteria) population ratio (M/A) were investigated as key microbiological and operating variables of VFA anaerobic degradations. M/A did not affect the size distribution and had little effect on extracellular polymer contents of the granules. Granular sludge with close spatial microbial proximity enhanced syntrophic degradation of VFAs compared to other cultures, such as suspended cultures. Optimum conditions were found to be propionate = 1.93 g/L, butyrate = 2.15 g/L, acetate = 2.50 g/L, HRT = 22 h, and M/A = 2.5 corresponding to maximum VFA removal and biogas production rate. Results of verification experiments and predicted values from fitted correlations were in close agreement at the 95% confidence interval. Granules seemed to be smaller particles and less stable in construction with an irregular fractured surface compared to the original granules.

Keywords: anaerobic syntrophic digestion, acetogenesis, methanogenesis, enriched cultures, granulation, response surface methodology (RSM)

1. Introduction

Anaerobic digestion, from the microbiology point of view, follows four major steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis [1]. Syntophic bacteria (propionate- and butyrate-degrading bacteria or H_2 producing bacteria), acetate oxidizing syntrophs and methanogens (hydrogenotrophs) form a special interrelated connection termed a syntrophic interaction [2,3]. Propionate and butyrate are the most important intermediates in the syntrophic reactions. Because of the thermodynamic restrictions, their degradations are regarded as the rate-limiting steps in anaerobic digestion. The anaerobic oxidation reaction of propionate and butyrate to acetate, CO_2 and H_2 is highly endergonic ($\Delta G^{o}_{Propionate}$= +76.1 and $\Delta G^{o}_{Butyrate}$= +48.1 kJ/mol both at 25°C and pH 7.0) and does not occur naturally. These reactions can, however, be accomplished through the syntrophic cooperation of H_2-producing (propionate- and butyrate-oxidizing) bacteria and H_2/formate-scavenging partners, which maintain a low H_2 partial pressure [2,3].

Volatile fatty acid (VFA) conversion studies have focused mainly on the syntrophic association of syntrophic bacteria with methanogens undergoing co-cultivation [4-6].

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These studies mainly investigated the effect of all VFAs, especially propionate, on the activity of syntrophic bacteria and methanogens. Also, some investigators have described the toxic effects of VFAs in the anaerobic digestion process, but the extent of this inhibition has not been precisely studied [7-10]. Previous studies have used VFAs with low loads on one of the VFAs, especially propionate, such as 0.8 g propionate/L [11], 3.0 g propionate/L [7], and 1.6 g propionate/L [12]. Recently, anaerobic degradation of only a high concentration of propionate individually as the sole carbon source in neutral pH was studied [13].

We previously investigated the syntrophic anaerobic digestion of VFAs (acetate, propionate and butyrate) in batch [14] and continuous stirred [15] reactors. The enriched cultures (syntrophic and methanogenic sludge) were cultivated in suspended growth and the results showed that distance between syntrophic bacteria (as producers) and methanogens (as consumers) was not highly suitable for trouble-free diffusion of intermediate metabolites (*e.g.*, formate/H_2). However, increasing the ratio of acetate oxidizing syntrophs and methanogen (hydrogenotrophs) to syntrophic bacteria (propionate- and butyrate-oxidizing bacteria) population (M/A) in a defined range, had a positive effect on the performance of the anaerobic syntrophic digestion of VFAs and biogas production rate (BPR). Attempts to decrease the distance between syntrophic bacteria and methanogens, and to minimize resistance to efficient mass transfer of metabolites could be performed through the construction of structured organized microbial agglomerates like granules [16]. Upflow anaerobic sludge bed (UASB) processes are based on the development of granules formed by the natural self-immobilization of the anaerobic microorganisms [17]. Also, the microstructure allows close microbial consortia proximity, optimal interspecies distances for syntrophic substrate transfer and diffusion limitations provide durability to process shocks and toxins [18].

The main objective of this research was to investigate, analyze and model the granular sludge process of VFA syntrophic anaerobic degradation and the performance of the enriched syntrophic and methanogenic microorganisms under mesophilic (37°C) conditions. This analysis aimed at finding important parameters in anaerobic digestion of VFAs and identifying their interactions. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response [19]. Recently, RSM has been applied to analyze, optimize and evaluate the interactive effects of independent variables in several chemical, biochemical and bioenvironmental processes [20-26]. However, its application to the analysis and modeling of syntrophic anaerobic degradation of VFAs was only reported in our previous studies [14,15]. In this study, RSM was used to analyze and model the process with respect to the simultaneous effects of five microbiological and operating variables [propionate, butyrate, acetate, M/A and hydraulic retention time (HRT)], and four parameters were assessed as responses. The significant factors and a continuous response surface of the main parameters were developed to yield an optimal region that satisfied the process specifications.

The present study provides valuable information about the interrelations of quality and process parameters at different values of microbiological and operating variables. Also, this research reveals the effects of syntrophic reactions and M/A on UASB reactor granule properties such as size distribution and extracellular polymer (ECP) contents.

2. Materials and Methods

2.1. Inocula

Enriched syntrophic bacteria (propionate- and butyrate-degrading bacteria) and methanogens (hydrogenotrophs) and acetate-oxidizing syntrophs cultures were used as the inocula. Syntrophic bacteria, acetate-oxidizing syntrophs and methanogens were enriched from granular sludge (pH, 7.4; volatile suspended solid (VSS), 67.2 g/L; total suspended solid (TSS), 92.4 g/L) from a dairy wastewater UASB reactor. The enrichment processes were described in our previous publications [14,15]. The concentrations of the propionate-degrading bacteria in the enriched cultures were VSS = 68.4 g/L and TSS = 78.2 g/L. For butyrate-degrading bacteria, the concentrations were VSS = 71.2 g/L and TSS = 81.3 g/L; and for acetate-oxidizing syntrophs and hydrogenotrophs, they were VSS = 74.3 g/L and TSS = 84.5 g/L.

2.2. Synthetic wastewater

Propionate, butyrate and acetate (99%, Merck, Germany) were diluted in tap water to achieve synthetic wastewater with desired chemical oxygen demand (COD) levels (low, 5.0 mg COD/L to high, 12.0 g COD/L). The COD:N:P ratio was maintained at 100:4:1 [27] by adding $NaNO_3$ and KH_2PO_4 as nitrogen and phosphorus sources, respectively. Oxygen was removed by N_2 sparging for 10 min before feeding to the bioreactor and a balloon containing the N_2 gas was placed on the feed reservoir to prevent oxygen entering into the feed vessel. The pH of the feed and within the reactor was not regulated throughout the experiment and was 4.5 ~ 5.5, corresponding to high and low loads, respectively. To increase the alkalinity, 4 g of $NaHCO_3$ was added per 1 L of feed.

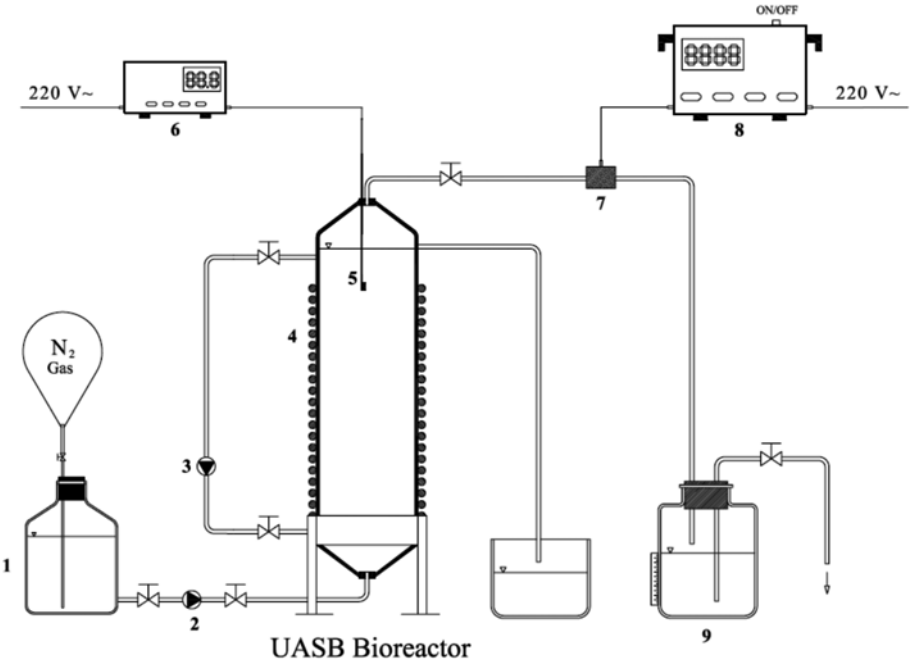

Fig. 1. Schematic flow diagram of the experimental set-up. (1) Feed reservoir, (2) feed peristaltic pump, (3) circulation peristaltic pump, (4) electrical heating tape, (5) temperature probe, (6) temperature controller, (7) methane sensor, (8) methane sensor transmitter, and (9) biogas collecting vessel.

2.3. UASB reactor and operating conditions

The UASB reactor was a glass cylinder with a diameter of 100 mm, height of 250 mm, and 2 L working volume (Fig. 1). At start up, the reactor was seeded by adding 400 mL of the enriched methanogen and syntrophic cultures, which was taken as their VSS concentrations (with defined M/A ratio; 1 ~ 3 g VSS of enriched methanogenic sludge to g VSS of enriched syntrophic sludge). The temperature was monitored with a probe connected to a transmitter and was maintained at 37 ± 1°C with an electrical heating tape (heating capacity: 40 W/m) attached to the outside surface of the reactor. To ensure efficient transfer of the intermediates and to release gas bubbles trapped in the medium, circulation was performed by peristaltic pump with a recycle ratio of 10 for 5 min per every 10 h. The produced biogas was collected by the water-displacement method. The concentrations of propionate, butyrate and acetate, HRT and M/A were selected as the primary factors to investigate the syntrophic anaerobic digestion of VFAs and central composite design (CCD) was used to design the experiments. The levels of the factors are shown in Table 1. Each factor was varied at five levels, while the other parameters were kept constant. Consequently, 47 experiments were conducted: 32 were organized in a full factorial design and 10 were related to axial points. The remaining five experiments involved repetition of the central design to obtain a good estimate of the experimental error. Experiments were designed by Design Expert Software (State-Ease, version 7.0.0).

2.4. Analytical methods

2.4.1. Methane concentration

CH_4 content in biogas were determined with a model TGS 2611 methane sensor (FIGARO, USA).

2.4.2. VFAs

Analyses of liquid reactor samples were conducted after centrifugation at 12,000 × *g* for 15 min and for acidification of the supernatant 500 μL of 1.0 N HCl was added to the samples. Propionate, acetate and butyrate were quantified using a model 7890 gas chromatograph (Agilent, USA) equipped with an auto-injector (7683 B series), a flame ionization detector (FID; H_2 flow rate: 35 mL/min, air flow rate: 350 mL/min) and a Chrompack Cp-Wax 52 CB fused-silica column (25 m × 0.32 mm i.d. and 0.2 μm film thickness). The injector and detector temperatures were maintained at 240 and 280°C, respectively. Helium (He) was used as the carrier gas at a flow rate of 3 mL/min and makeup flow rate of 5 mL/min. The oven temperature was programmed at 40°C for 4 min, raised to 180°C at 30°C/min, and then held at 180°C for 1 min.

2.4.3. Scanning electron microscopy (SEM)

The granules fixed for SEM were washed three times for 20 min each in 0.1 M cacodylate buffer. Dimethyl sulf-

Table 1. The levels of factors in the experiments based on central composite design (CCD)

Factor	Low axial	Low factorial	Center	High factorial	High axial
	(–α)	(–1)	(0)	(+1)	(+α)
A: HRT (h)	11.5	17	21	25	30.5
B: M/A (g VVS/g VVS)	1.1	1.7	2.1	2.5	3.1
C: Propionate concentration (g/L)	0.10	0.94	1.54	2.15	2.99
D: Butyrate concentration (g/L)	0.0	1.16	2.00	2.84	4.00
E: Acetate concentration (g/L)	0.0	1.45	2.50	3.55	5.00

HRT: hydraulic retention time; M/A: acetate-oxidizing syntrophs and hydrohenotrophs to syntrophic bacteria ratio; VVS: volatile suspended solids.

oxide (DSMO) was infiltrated at 30% for 30 min followed by 50% for 1 h. The granules were fast frozen in an aluminum block in liquid nitrogen. The granules were dehydrated in 30, 50, 60, 70, 80, 90, and 100% water-ethanol series before thawing once in hexamethyl disilazane (HMDS) and leaving in HMDS overnight for evaporation. The granules were viewed using a TESCAN SEM with accelerating voltage of 20 kV [28].

2.4.4. Size distribution

Granules were placed in a flat glass dish on a light table and the size distribution analyzed by image analysis equipment [29].

2.4.5. Total ECP analysis

ECP extraction was performed by a cation exchange resin (CER) method [29]. The granules were gently crushed in a polyethylene bag until they were a fine paste. The crushed granules were re-suspended in buffer solution (2 mM Na_3PO_4, 4 mM NaH_2PO_4, 9 mM NaCl, and 1 mM KCl) at a concentration of 3.0 ~ 4.0 g/L VSS and extracted in the same cell as used for the shear strength characterization at 700 rpm. Dowex 50 8 20 ~ 50 mesh CER in the sodium form (Sigma-Aldrich Chemicals, Australia), washed in buffer at a mass of 70 g/g VS. Extraction was at 4°C. Total ECP levels were measured during extraction by COD analysis.

2.4.6. VSS, TSS, COD, and pH

Determinations were made using standard methods [30]. pH was measured using a model 620 pH meter (Metrohm, Germany).

3. Results and Discussion

3.1. Granule properties

Effects of syntrophic substrates, enrichment and amount of M/A on the granule size distributions were investigated. The size distributions for granules at different M/A as well as original granular sludge are shown in Fig. 2. Increasing M/A (*i.e.*, increasing the methanogenic population) did not significantly affect the size distributions of the granules (Figs. 2A, 2B, 2C, 2D, and –2E) and most of the syntrophic granules in the UASB reactor exceeded 1 mm in diameter. The size of the original granules that was mainly due to the substrate type (most > 2.5 mm) was considerably greater than the enriched granular sludge. According to the literature, granules cultivated on acidified substrates, such as VFAs, are generally smaller than granules cultivated on acidogenic substrates [31,32].

ECPs play an essential role in maintaining the spatial structural integrity of the microbial matrix, which they can first extract from the hydrogenotrophs. Also, increasing in-reactor acidification increases ECP production [32]. In this study, the effects of M/A on the total ECP contents of the granules in the syntrophic anaerobic digestion of VFAs were studied. Total ECP concentrations for different granules (different M/As) are shown in Fig. 3. When M/A was increased, the methanogenic population was higher than the syntrophic bacteria; indeed, the number of hydrogenotrophs (as ECP producers) increased. Therefore, total ECP concentrations must be greater in the higher M/A ranges [3]. But, Fig. 3 shows that with increasing M/A, total ECP was only marginally increased. For example, total ECP (after 40 h extraction) for granules at M/A = 1.1 was < 0.18 mg COD/mg VSS, while at M/A = 3.1 was > 0.25 mg COD/mg VSS. As a result, it could be concluded that increasing methanognic population (about 3 times), especially hydrogenotrophs, did not appreciably affect ECP production in the granules.

The structures of the original and enriched granules seemed to differ. SEM of the original and enriched granules is shown in Fig. 4. Original granules were more stable, packed with smooth surfaces and apparently had more density, while enriched granules seemed to be less stable with broken portions, and with a light and irregular surface. The reason might be attributed to granules formed and subjected to acidogenic products (VFAs) and low pH conditions [33].

3.2. Statistical analysis

Forty-seven experiments were designed using CCD. The

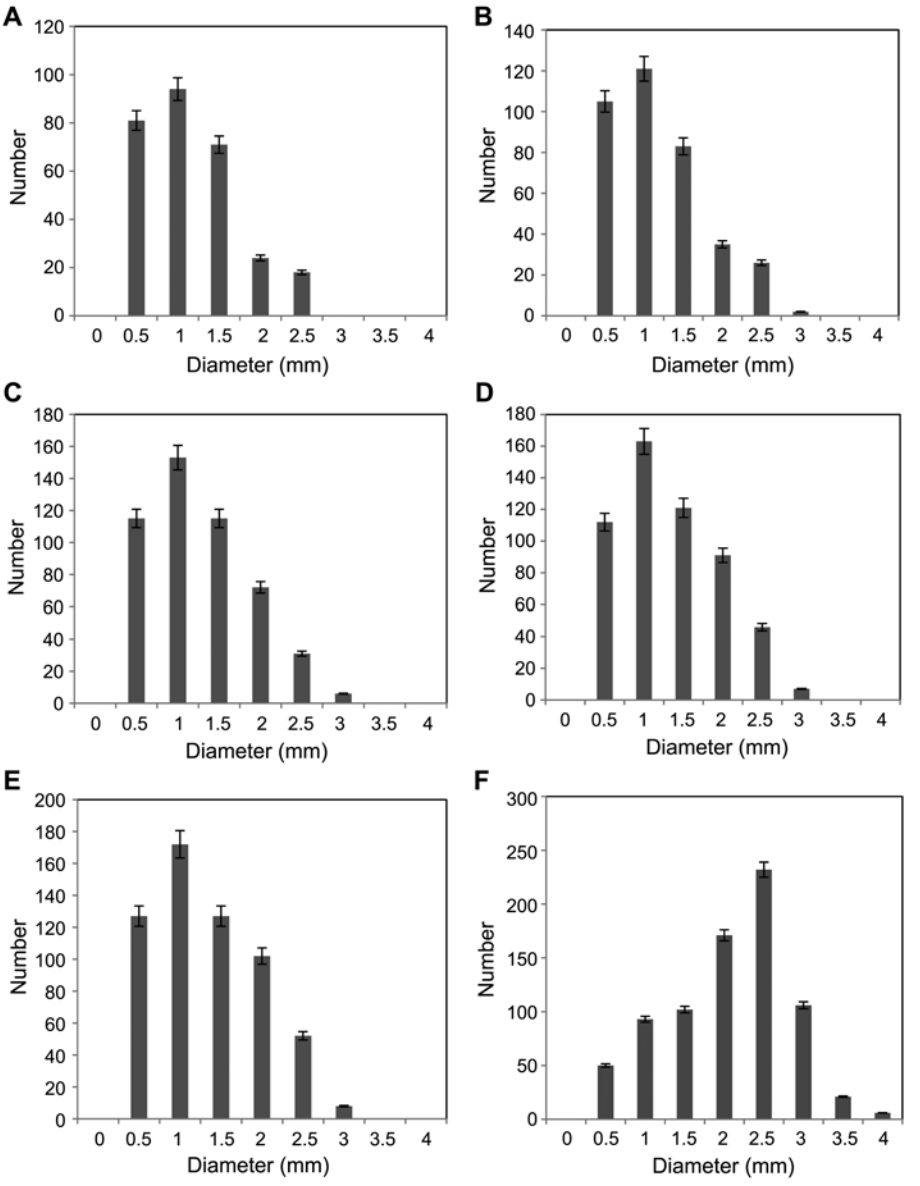

Fig. 2. Size distributions of granules at (A) M/A = 1.1, (B) M/A = 1.7, (C) M/A = 2.1, (D) M/A = 2.5, (E) M/A = 3.1, and (F) original granules.

experimental conditions and their responses for mesophilic anaerobic digestion processes in UASB reactor are shown in Table 2. The data were fitted to quadratic correlations, and then adequate correlations were found to predict the response variables. Analysis of variance (ANOVA) results for the responses are summarized in Table 3. The quality of the fit of quadratic correlations was expressed by the coefficient of determination (R^2). The relatively high R^2 values indicated that the quadratic equations for the effluent propionate, butyrate, acetate and BPR were very capable of representing the system under the given experimental domain.

According to the data in Table 3, the fitted correlations were significant at the 95% confidence interval. The correlation statistic significance was checked by the *F*-test for lack of fit using appropriate software [19]. The lack of fit F-statistics were not statistically significant because the *p*-values were > 0.05. Adequate precision is a measure of the range of the predicted response relative to its associated error or, in other words, a signal-to-noise ratio. Its desired value is four or more [34]. The values were found to be desirable for the four correlations (see Table 3; in the adequate precision column, the values are much greater than four). Simultaneously, low response values for the coefficients of variation (CVs) indicated good accuracy and dependability of the experiments.

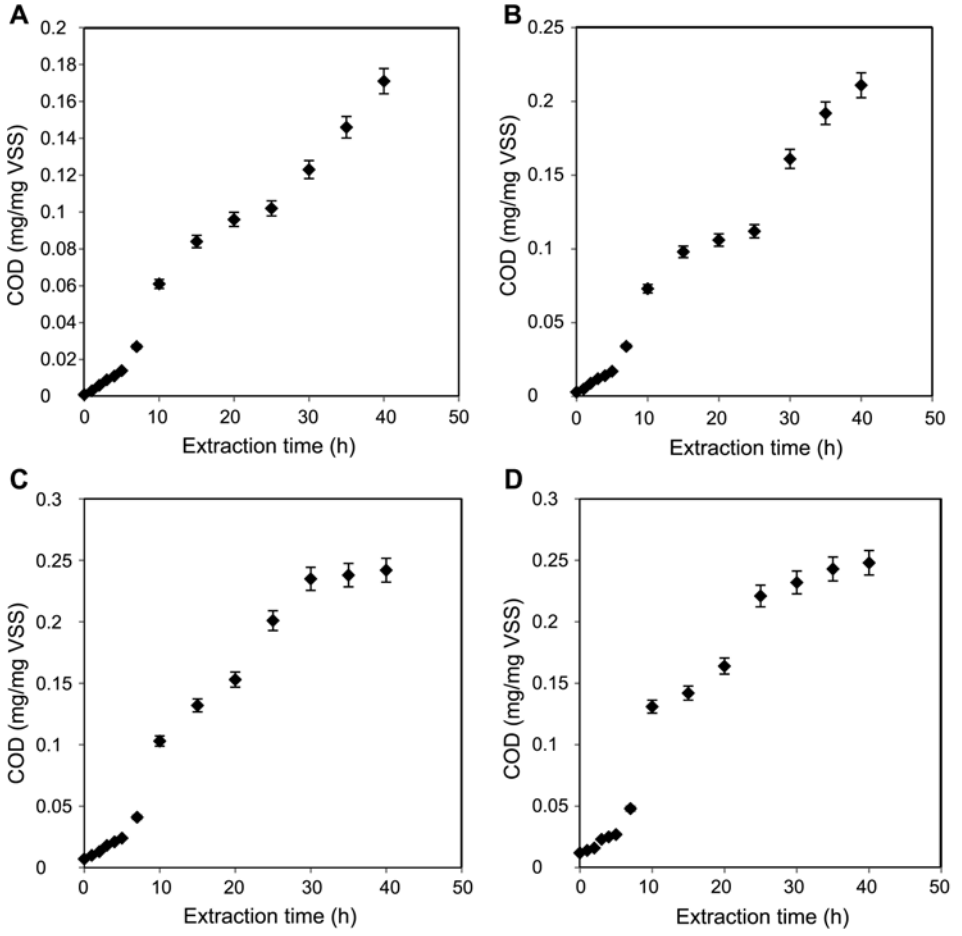

Fig. 3. Total ECP contents of granules at (A) M/A = 1.1, (B) M/A = 1.7, (C) M/A = 2.5, and (D) M/A = 3.1.

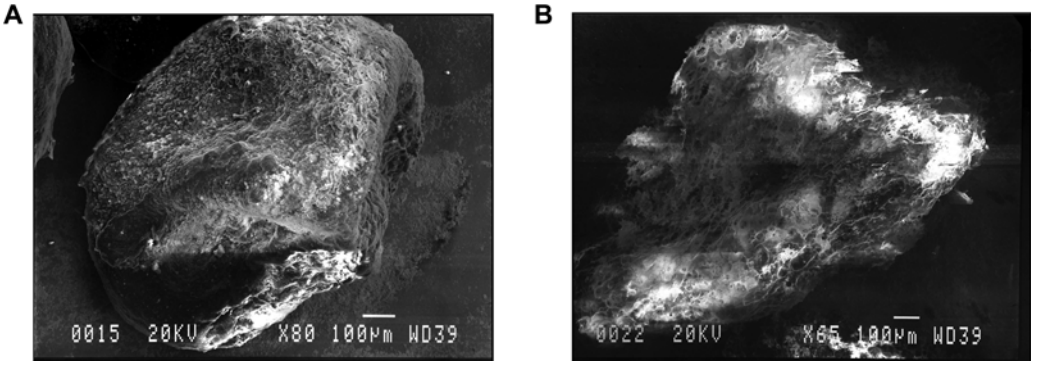

Fig. 4. Scanning electron micrographs of (A) original and (B) enriched granules.

3.3. Effects of M/A and HRT on VFA removal and BPR

Anaerobic digestion of propionate and butyrate is highly endergonic and does not occur naturally (in view of thermodynamic principles) in the anaerobic digesters [2]. A small population of the methanogens will not be able to metabolize the hydrogen and acetate produced by the syntrophic bacteria. Increasing the methanogenic population could be used as a method to promote the efficient completion of these reactions [35].

In our study, the ratio of M/A was increased from about 1 ~ 3 to investigate the effect of the methanogenic population on the syntrophic anaerobic process. Table 2 shows when the M/A was increased from 1 to 2.1, the removal rates of propionate, butyrate and acetate were increased. However, when the M/A was 3.1, the removal rates were decreased. This showed that at the very high M/

Table 2. The experimental plan of anaerobic digestion of volatile fatty acids (VFAs) and their raw responses results in the UASB reactor (effluent acetate, propionate, butyrate, and BPR)

Run	Factors					Responses			
	HRT (h)	M/A	Propionate (g/L)	Butyrate (g/L)	Acetate (g/L)	Propionate (g/L)	Butyrate (g/L)	Acetate (g/L)	BPR (mL/L/h)
1	21(0)*	1.1(–α)*	1.54(0)*	2.00(0)*	2.50(+1)*	0.57	0.93	1.25	85
2	25(+1)	1.7(–1)	0.94(–1)	1.16(–1)	1.45(–1)	0.27	0.44	0.84	46
3	25(+1)	1.7(–1)	2.15(+1)	1.16(–1)	1.45(–1)	0.99	0.56	0.74	57
4	25(+1)	1.7(–1)	0.94(–1)	1.16(–1)	3.55(+1)	0.38	0.58	1.92	58
5	25(+1)	1.7(–1)	0.94(–1)	2.84(+1)	1.45(–1)	0.42	1.18	0.74	70
6	25(+1)	1.7(–1)	2.15(+1)	1.16(–1)	3.55(+1)	1.02	0.57	1.97	69
7	25(+1)	1.7(–1)	2.15(+1)	2.84(+1)	1.45(–1)	1.13	1.31	0.65	80
8	25(+1)	1.7(–1)	0.94(–1)	2.84(+1)	3.55(+1)	0.49	1.93	2.67	52
9	25(+1)	1.7(–1)	2.15(+1)	2.84(+1)	3.55(+1)	1.53	1.87	2.70	57
10	17(–1)	1.7(–1)	0.94(–1)	1.16(–1)	1.45(–1)	0.35	0.58	1.01	53
11	17(–1)	1.7(–1)	2.15(+1)	1.16(–1)	1.45(–1)	1.21	0.74	0.97	61
12	17(–1)	1.7(–1)	0.94(–1)	1.16(–1)	3.55(+1)	0.47	0.76	2.08	69
13	17(–1)	1.7(–1)	0.94(–1)	2.84(+1)	1.45(–1)	0.56	1.35	1.01	85
14	17(–1)	1.7(–1)	2.15(+1)	1.16(–1)	3.55(+1)	1.27	0.75	2.31	82
15	17(–1)	1.7(–1)	2.15(+1)	2.84(+1)	1.45(–1)	1.41	1.50	0.87	95
16	17(–1)	1.7(–1)	0.94(–1)	2.84(+1)	3.55(+1)	0.65	2.14	2.90	56
17	17(–1)	1.7(–1)	2.15(+1)	2.84(+1)	3.55(+1)	1.65	2.02	2.99	65
18	30.5(+α)	2.1(0)	1.54(0)	2.00(0)	2.50(0)	0.40	0.55	0.65	84
19	21(0)	2.1(0)	1.54(0)	0.00(–α)	2.50(0)	0.32	0.00	0.57	75
20	21(0)	2.1(0)	1.54(0)	2.00(0)	0.00(–α)	0.35	0.65	0.34	74
21	21(0)	2.1(0)	0.10(–α)	2.00(0)	2.50(0)	0.05	0.54	0.68	87
22	21(0)	2.1(0)	1.54(0)	2.00(0)	2.50(0)	0.48	0.73	0.84	105
23	21(0)	2.1(0)	1.54(0)	2.00(0)	2.50(0)	0.49	0.76	0.82	104
24	21(0)	2.1(0)	1.54(0)	2.00(0)	2.50(0)	0.46	0.71	0.83	110
25	21(0)	2.1(0)	1.54(0)	2.00(0)	2.50(0)	0.50	0.78	0.85	106
26	21(0)	2.1(0)	1.54(0)	2.00(0)	2.50(0)	0.44	0.71	0.81	110
27	21(0)	2.1(0)	2.99(+α)	2.00(0)	2.50(0)	1.39	1.42	1.17	90
28	21(0)	2.1(0)	1.54(0)	4.00(+α)	2.50(0)	1.02	2.99	1.55	70
29	21(0)	2.1(0)	1.54(0)	2.00(0)	5.00(+α)	0.97	1.39	3.09	75
30	11.5(–α)	2.1(0)	1.54(0)	2.00(0)	2.50(0)	0.76	1.07	1.02	150
31	25(+1)	2.5(+1)	0.94(–1)	1.16(–1)	1.45(–1)	0.17	0.35	0.66	56
32	25(+1)	2.5(+1)	2.15(+1)	1.16(–1)	1.45(–1)	0.71	0.44	0.63	70
33	25(+1)	2.5(+1)	0.94(–1)	1.16(–1)	3.55(+1)	0.25	0.49	1.73	68
34	25(+1)	2.5(+1)	0.94(–1)	2.84(+1)	1.45(–1)	0.30	0.86	0.58	87
35	25(+1)	2.5(+1)	2.15(+1)	1.16(–1)	3.55(+1)	0.74	0.47	1.53	89
36	25(+1)	2.5(+1)	2.15(+1)	2.84(+1)	1.45(–1)	0.80	0.96	0.52	100
37	25(+1)	2.5(+1)	0.94(–1)	2.84(+1)	3.55(+1)	0.44	1.75	2.54	62
38	25(+1)	2.5(+1)	2.15(+1)	2.84(+1)	3.55(+1)	1.47	1.79	2.62	65
39	17(–1)	2.5(+1)	0.94(–1)	1.16(–1)	1.45(–1)	0.20	0.43	0.87	73
40	17(–1)	2.5(+1)	2.15(+1)	1.16(–1)	1.45(–1)	0.96	0.53	0.78	86
41	17(–1)	2.5(+1)	0.94(–1)	1.16(–1)	3.55(+1)	0.30	0.61	1.90	87
42	17(–1)	2.5(+1)	0.94(–1)	2.84(+1)	1.45(–1)	0.40	1.17	0.76	108
43	17(–1)	2.5(+1)	2.15(+1)	1.16(–1)	3.55(+1)	0.90	0.57	1.85	112
44	17(–1)	2.5(+1)	2.15(+1)	2.84(+1)	1.45(–1)	1.09	1.26	0.63	123
45	17(–1)	2.5(+1)	0.94(–1)	2.84(+1)	3.55(–1)	0.49	1.95	2.78	76
46	17(–1)	2.5(+1)	2.15(+1)	2.84(+1)	3.55(–1)	1.59	1.82	2.78	80
47	21(0)	3.1(+α)	1.54(0)	2.00(0)	2.50(0)	0.56	0.81	0.90	100

HRT: hydraulic retention time; M/A: acetate-oxidizing syntrophs and hydrohenotrophs to syntrophic bacteria ratio; VVS: volatile suspended solids; BPR: biogas production rate.
*Level code values of the parameters.

Table 3. Analysis of variance (ANOVA) results for the correlations from DX-7 for the studied responses in the UASB reactor (effluent acetate, propionate, butyrate, and BPR)

Response	Correlations with significant terms	*P*-value	R^2	Adj. R^2	SD	Adequate precision	CV
Propionate	$504.1 - 74.8A - 69.0B + 357.9C + 136.1D + 94.6E - 30.3AC + 12.0AE - 28.0BC + 14.5BD + 12.8BE + 46.9CD + 33.1CE + 54.3DE + 34.2A^2 + 31.8B^2 + 58.8C^2 + 50.7D^2 + 48.52E^2$	< 0.0001	0.9352	0.8853	143.6	17.7	20.1
Butyrate	$750.9 - 88.9A - 71.9B + 62.2C + 533.5D + 188.0E - 15.1AD + 10.1AE - 20.1BD + 14.3BE - 41.7CE + 154.0DE + 17.5A^2 + 28.8B^2 + 48.5C^2 + 138.8D^2 + 55.4E^2$	< 0.0001	0.9707	0.9482	138.1	28.0	13.6
Acetate	$925.7 - 98.9A - 93.9B + 16.0C + 191.1D + 727.5E - 11.9AE - 15.8BC + 18.1BD - 12.9BE + 29.5CE + 230.9DE + 48.2A^2 + 90.5B^2 + 64.6C^2 + 87.7D^2 + 230.9E^2$	< 0.0001	0.9228	0.8635	306.7	22.2	16.9
BPR	$103.7 - 8.8A + 7.5B + 4.4C + 2.6D - 2.3E - 2.2 + 1.0BC - 0.8BE - 1.5CD - 11.5DE - 4.2B^2 - 4.9C^2 - 7.8D^2 - 7.4E^2$	< 0.0001	0.8150	0.6727	12.2	9.5	15.0

R^2: determination coefficient, Adj. R^2: adjusted R^2, SD: standard deviation, CV: coefficient variation; BPR: biogas production rate.

A ranges, the H_2-producing bacteria (propionate- and butyrate-oxidizing bacteria) were not sufficient to degrade the high concentrations of propionate and butyrate. Under these conditions, high levels of propionate and butyrate inhibited the acetogenic reactions and suppressed the growth of syntrophic bacteria. Consequently, their degradation was slow. Because methanogenic conversion of acetate does occur freely (exergonic, $\Delta G^{o}_{Acetate} = -72.2$ kJ/mol) and is independent from the other VFA conversions, its removal rate was increased sensibly as the M/A increased. Accordingly, in the high M/A ranges, acetate-oxidizing syntrophs were the most active anaerobes among the anaerobic digesters. Syntrophic studies may be more accurate when the M/A ratios become more accurate. Consequently, degradation of propionate and butyrate were slow. Methanogenic conversion of acetate is exergonic, therefore its oxidation must occur thermodynamically and be independent from degradation of other VFAs. In spite of the previously documented occurrences in the continuous stirred reactor with suspended growth [15], in the UASB reactor we observed that syntrophic anaerobic degradation of acetate was reduced in the higher M/A (3.1) ranges. In higher M/A ranges propionate and butyrate were accumulated at high concentrations and pH was decreased strongly. Consequently, the growth and activity of methanogens, especially acetate-oxidizing syntrophs due to their high sensitivity to low pH [35] was diminished or even repressed. Accordingly, in the high M/A ranges, the acetate-oxidizing syntrophs in the granular sludge like the syntrophic bacteria were not so active anaerobic microorganisms. Another reason for the reduction of acetate oxidation might be the inappropriate position of acetate-oxidizing syntrophs inside the granules in a manner that efficient diffusion acetate was restricted and they could not readily access their substrate.

The HRT had a positive effect on VFA removal. Increasing the HRT led anaerobic microorganisms to adapt to the low pH conditions, and removal efficiencies improved (see its coefficients in the relevant fitted correlations in Table 3). Similar results were obtained in our previous work in a continuous stirred reactor [15]. Since increasing the HRT led to a decreasing organic loading rate, BPR was decreased with the increase of HRT in the entire domains. Its coefficient in Table 3 confirmed this result (–8.8). On the other hand, in the continuous stirred reactor HRT in small narrow ranges had a positive effect on BPR. Higher rates of syntrophic reactions in the UASB reactor might be a reason for this little difference. In contrast, BPR at constant HRT increased with the M/A ratio. At higher M/A ratios, the methanogenic population was greater than the syntrophic population; therefore, acetate was immediately converted to CH_4 and CO_2. However, when the syntrophic population was very small, the BPR was reduced. Thus, in this situation, propionate and butyrate were not oxidized. According to the fitted correlations in Table 3, it could be concluded that the M/A affected the BPR significantly, because its coefficient was high (7.5).

As a practical result, when BPR was not acceptable, the improvement of the microbial condition of the anaerobic sludge involved is the only way to improve the anaerobic digestion efficiency. However, this would not be very practical in the operation of huge and full-scale anaerobic digesters because changing of the other operating variables, such as HRT and amounts of volatile fatty acids in the anaerobic digester does not improve the BPR.

3.4. Effect of external addition of VFAs on propionate and butyrate oxidations

The presence of VFAs leads to a pH drop in the digester; the greater the pH drop in the digester, the more pronounced is the VFA toxicity [36]. Because the syntrophic degradation of propionate and butyrate is extremely endergonic, its syntrophic anaerobic oxidation is thermodynamically repressed. In addition, anaerobic oxidation of propionate and butyrate is inhibited *via* a pH drop in the digester. On the other hand, the methanogenic degradation of acetate is exergonic; so, it could be expected that its inhibitory effect is due to the pH drop. This phenomenon decreases the growth of methanogens, although acetate promotes propionate oxidation thermodynamically. In other words, the negative effect of a pH drop caused by acetate on propionate oxidation is greater than their positive thermodynamical effect. Fig. 5 shows the effects of butyrate and acetate on the anaerobic oxidation of propionate. This figure shows that acetate in a moderate concentration ranges (around 1.50 ~ 1.90 g/L) had a bit more of a positive effect on propionate degradation. But generally, when concentrations of butyrate and acetate were increased, propionate removal was decreased. The positive effect of acetate on propionate oxidation is attributed to its thermodynamic energetic at low concentration when pH is still moderate.

Furthermore, because the mechanisms of butyrate and acetate inhibition (thermodynamically and *via* pH drop) were rather different, their observed inhibitory effects on propionate removal were also dissimilar. The coefficient of the butyrate (136) term in the fitted correlation in Table 3 was higher than the acetate coefficient (95). Consequently, the inhibitory level of butyrate was considerably higher than acetate inhibitory effect. Contrarily, in the continuous stirred reactor (suspended growth), the inhibitory effect of acetate on propionate degradation was much higher than that of butyrate. This result revealed the important role of spatial microbial proximity (granular versus suspended cultures) as a main operational and microbiological factor in determination of interactions and magnitude of the variables. Indeed, spatial location of the acetate-oxidizing syntrophs might be more appropriate than the butyrate-degrading bacteria in the organized structure of the granules. Moreover, experimental results (Table 2) showed that the relative inhibitory effects of butyrate and acetate (in the suspended growth, acetate inhibition was lower at higher M/As) were not significantly dependent on the M/A ratios. The coefficient of propionate in the fitted correlation was 358, which was the highest value. Therefore, it can be concluded that propionate is the most inhibitory parameter affecting propionate removal.

The anaerobic oxidation of propionate was more unfavorable than that of butyrate, but the inhibition pattern of VFAs on the syntrophic anaerobic degradation of both was similar [16]. Propionate and acetate decreased the pH of the digester and, therefore, it could inhibit the growth of syntrophs (propionate- and butyrate-oxidizing bacteria) and methanogens [21]. In addition, thermodynamically, propionate hinders the anaerobic conversion of butyrate, whereas acetate enhances it. As a result, it could be expected that the inhibitory effect of propionate on butyrate degradation is much higher than the inhibitory effect of acetate. The effects of propionate and acetate on butyrate oxidation are shown in Fig. 6. The results showed that, in spite of what can be understood from the above statements, the inhibitory effect of acetate was greater than that of propionate. The coefficients of propionate (62) and acetate

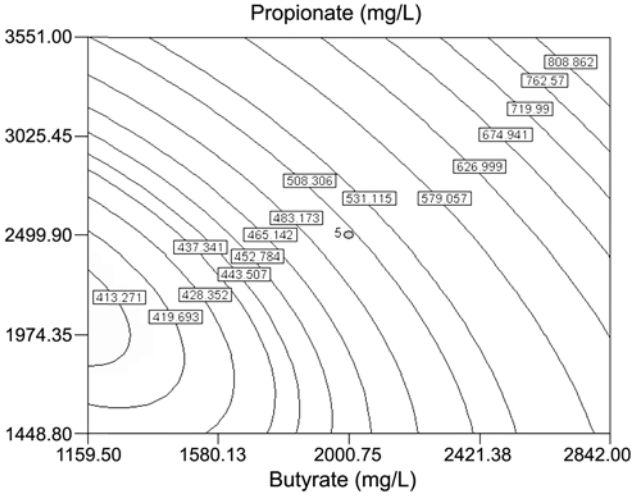

Fig. 5. The inhibitory effects and the interactions of acetic acid (acetate) and butyric acid (butyrate) on propionic acid (propionate) conversion in the UASB reactor.

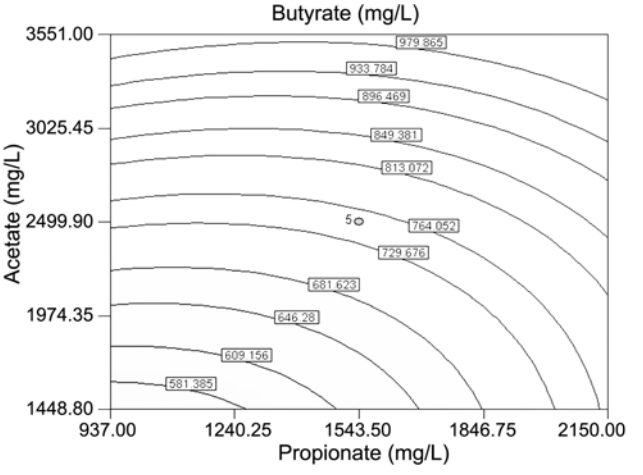

Fig. 6. The inhibitory effects and the interactions of propionic acid (propionate) and acetic acid (acetate) on butyric acid (butyrate) conversion in the UASB reactor.

(188) in the fitted correlation proved the above result. Similar result was observed in the continuous stirred reactor with suspended enriched syntrophic and methanogenic sludge [15]. The reason for this may be mostly attributed to the spatial position or juxtaposition of the syntrophic bacteria and methanogens in the granular culture because, in syntrophic cultures, diffusion of the intermediates (H_2/formate and acetate) from the syntrophic bacteria (as producers) to the methanogens (as consumers) is the main mechanism of syntrophic reactions [3,16]. Furthermore, a pH drop due to the presence of acetate could be another reason for higher inhibitory effect of acetate, because this inhibitory effect of acetate is steeper than its stimulatory thermodynamical effect. Similar to propionate, butyrate itself had the highest inhibitory effect on the removal of butyrate because its coefficient (534) in the fitted correlation was much higher than that of propionate or acetate. The results show that acetate, propionate and butyrate inhibit the anaerobic conversion of butyrate and also, butyrate itself has the highest inhibitory effect on butyrate degradation.

3.5. Effect of external addition of VFAs on acetate oxidation

Both propionate and butyrate inhibit the methanogenic conversion of acetate and obstruct the growth of acetate-oxidizing syntrophs [3,10,16,37]. Fig. 7 shows the presently-observed effects of propionate and butyrate on the anaerobic degradation of acetate. Propionate, although hindering (thermodynamically and via pH drop) the anaerobic oxidation of acetate, in some ranges (around 0.90 ~ 1.50 g/L) slightly stimulated the syntrophic anaerobic degradation of acetate in the UASB reactor. But, propionate with higher concentration ranges (> 1.50 g/L) inhibited the anaerobic oxidation reaction of acetate. However, as shown in Table 3, the inhibitory effect of propionate (its coefficient 16) on acetate oxidation was highly less than that of butyrate (its coefficient 191). The low inhibitory effect of propionate on acetate degradation not only was a thermodynamic phenomenon, but might have also been due to the possible proximity of syntrophic bacteria and methanogenic archaea located in the granular structures in the UASB reactor. Similar results were observed in the continuous stirred reactor (suspended culture) with intermittent minimal mixing. Acetate had the highest inhibitory effect on acetate oxidation, because its coefficient in the relevant fitted correlation was the highest (728).

3.6. Effects of VFAs on BPR

Since propionate, butyrate and acetate affect growth of all syntrophic microorganisms, especially methanogens, BPR was influenced by the VFAs levels. Hydrogenotrophic methanogens reduce CO_2 with H_2 and produce CH_4 and acetate-oxidizing syntrophs convert acetate to methane. Propionate at concentrations of 900 ~ 1,850 mg/L and butyrate at concentrations of 1.10 ~ 2.00 g/L had positive effects and enhanced the BPR. But, acetate at all ranges had an inhibitory effect on BPR. The coefficients of VFAs in the fitted correlation in Table 3 confirmed these results. From the thermodynamic point of view, the effect of propionate and butyrate on BPR is negative if only they were in their standard condition (298 ºK, 1 bar and all concentrations are 1 mole); however they could be favorable in their real concentrations in the anaerobic digestion process. Acetate is stronger than other VFAs; therefore it seems that its positive thermodynamic effect (always being exergonic)

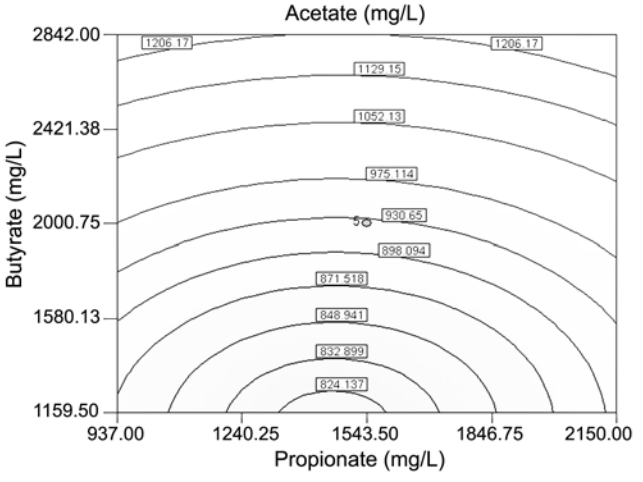

Fig. 7. The pattern of influences of propionic acid (propionate) and inhibitory effects of butyric acid (butyrate) on acetic acid (acetate) conversion in the UASB reactor.

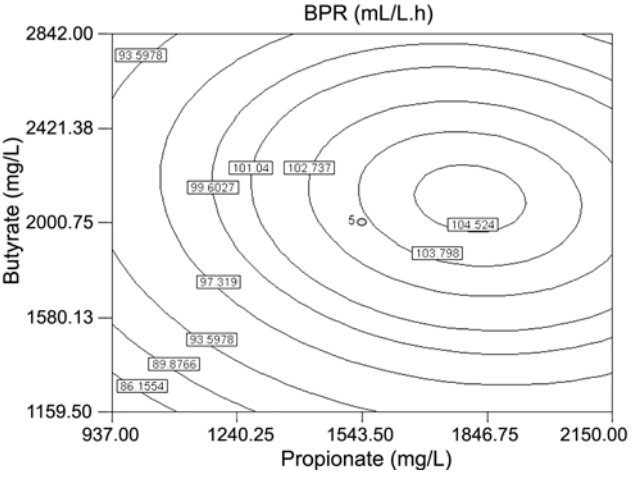

Fig. 8. The simulatory effects of propionic acid (propionate) and butyric acid (butyrate) on biogas production rate (BPR) for mesophilic syntrophic anaerobic digestion of volatile fatty acids (VFAs) in the UASB reactor.

Table 4. Verification experiment results at the optimum conditions (propionate = 1.93 g/L, butyrate = 2.15 g/L, acetate = 2.50 g/L, HRT = 21 h, and M/A = 2.5)

Response	Target	Correlation predicted	Confirmation experiment	Confidence interval (95%)	
				Low	High
Propionate (g/L)	Minimize	0.71	0.80	0.59	0.83
Butyrate (g/L)	Minimize	0.84	0.91	0.73	0.95
Acetate (g/L)	Minimize	0.96	1.14	0.70	1.21
BPR(mL/L/h)	Maximize	105	102	95	115

BPR: biogas production rate.

is suppressed by its extreme effect on pH drop. Also, the effect of propionate on BPR was higher than that of butyrate (Table 3). Fig. 8 illustrates the effects of propionate and butyrate on BPR.

Analysis of the biogas contents showed that the methane content (21 ~ 55%) of the biogas was influenced by the pH (5.0 ~ 6.9) of the digester. The biogas should be contained CH_4, CO_2 and H_2, therefore the presence of hydrogen (product of acetogenesis reactions) could be another evident for positive effects of propionate and butyrate on BPR. This demonstrates that the methanogenic activity of the methanogens, especially the acetate-oxidizing syntrophs, mainly depended on the pH. However, since hydrogenotrophs are more tolerant of acidic conditions than the acetate-oxidizing syntrophs, observed methanogenic activity at a lower pH can be attributed to hydrogenotrophs [38]. It should be mentioned that, generally, syntrophic bacteria are more resistant against pH drop; as a result, hydrogenation is not slowed down in acidic conditions. On the other hand, the weak methanogenic activity observed in the acidic condition was not related to the activity of acetate-oxidizing syntrophs, but is due to the restricted activity of hydrogenotrophs. The VFAs affected the BPR with a similar manner (qualitatively) in the continuous stirred reactor (suspended growth), but the performance of this process and biogas production rates was sensibly lower [15].

3.7. Maximum VFA removal and BPR

According to the main target (maximum VFA removal and BPR), the optimum conditions obtained were propionate = 1.93 g/L, butyrate = 2.15 g/L, acetate = 2.50 g/L, HRT = 22 h, and M/A = 2.5. To check the accuracy of the fitted correlations at the 95% confidence interval of the optimum conditions, the UASB reactor was operated accordingly to compare the actual and predicted responses. Table 4 presents the results of the experiment conducted at the optimum conditions and showed that verification experiments and predicted values from fitted correlations were in close agreement at a 95% confidence interval. The accuracy of the optimum conditions used in design of the experiments was checked to verify that the experimental finding was in close agreement with the predictive values (Table 4). Comparison of this results with the results obtained (optimum conditions were propionate = 1.13 g/L, butyrate = 1.83 g/L, acetate = 1.73 g/L, HRT = 21 h, and M/A = 2.5) in the continuous stirred reactor [15] revealed that the UASB reactor with granular sludge was highly more efficient than the suspended cultures, consequently, distances between syntrophic microorganisms in organized structure of the granules were optimized to minimize the resistances against efficient diffusion and transfer of substrates and intermediate metabolites.

4. Conclusion

HRT and M/A has positive effects on VFA removal and BPR. However, performance at very high M/A values is drastically decreased. M/A was the most important factor that affected BPR. All VFAs inhibit the VFA removal and the effect of butyrate on VFA removal is more significant. The distance between syntrophic organisms plays an important role in syntrophic reactions and granular sludge provides more efficient spatial microbial proximity than suspended culture to enhance the flux of substrates and metabolites. Also, syntrophic studies may be more accurate when the M/A ratios become more accurate and this goal may be achieved by establishing optimum ratios of the propionate-degrading bacteria to butyrate-degrading bacteria in the syntrophic population.

Acknowledgement

The authors are grateful to State-Ease, Minneapolis, MN, USA, for the provision of the Design-Expert package.

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