Effect of initial pH on mesophilic hydrolysis and acidification of swine manure

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Abstract Effects of initial pH (3–12) on mesophilic hydrolysis and acidification reactions of swine manure was studied. The initial pH changed the microbial community in the suspension so as to affect hydrolysis and acidification reactions on swine manure. At pH 10-12 the *Clostridium alkalicellum* and/or *Corynebacterium humireducens* were enriched and the soluble chemical oxygen demand (SCOD), total volatile fatty acids (VFAs), proteins and carbohydrates from manure were increased in quantities. In particular, at pH 10 the VFA concentration peaked at 13600 mg-COD/L, with acetate and propionate accounting for 71.8% of the total VFAs. Acidic environment facilitates release of ammonium from manure. The *Butyricimonas* sp. was found existing at initial pH 5 which led to accumulated quantities of butyrate. Initial pH adjustment was revealed to be an effective way to manipulate rates and end products of hydrolysis and acidification of swine manure.

Keywords: Swine manure; Hydrolysis; Acidification; pH; Volatile fatty acids; Microbial community

1. Introduction

Hydrolysis and acidification reactions can convert particulate organic polymers to volatile fatty acids (VFAs), mainly including acetate (Ac), propionate (Pr), n/iso-butyrate (n/iso-Bu) and n/iso-valerate (n/iso-Va). The so-yielded VFAs are applicable for production of biodegradable plastics (Satoh *et al.*, 1998), as carbon source for biological nutrient removal reactions (Moser-Engeler *et al*., 1998), and as substrates for biogas production (Weiland, 2010). Amongst the VFAs, the Ac is readily utilized by numerous microorganisms (Wang *et al.*, 1999) and is a valuable chemical in markets (Padma, 1999).

The yields and distributions of hydrolysis and acidification products from particulate organic polymers are influenced by incorporated microbial communities (Zhang *et al.*, 2012), adopted pretreatment methods (Pilli *et al*., 2011) and operational conditions (Zhang *et al*., 2005). The culture pH affects the activities of specific acidogenic microbial populations (Horiuchi *et al*., 1999; Zhang *et al*., 2012) and of methanogenic bacteria (Ghosh *et al*., 2000), so affecting the accumulation of yielded VFAs. Chen *et al.* (2007) adjusted initial pH to 10 for activated sludge and noted a high yield of VFAs. Zhang *et al.* (2005) noted that at pH 7 high levels of VFAs can be produced from kitchen wastes. Horiuchi *et al.* (2002) reported that increase in pH from 5 to 8 in cultivated sludge shifts the produced VFAs from Bu to Ac and Pr.

The swine manure contributes more than 15% of national COD pollution in China (CMEP, 2011). However, until now few works were conducted to explore effects of culture pH on hydrolysis and acidification of swine manure. The purpose of this work is to investigate the effects of initial pH of swine manure on the accumulation of total VFAs, particularly on the maximum yields of acetate in suspension, and to disclose

the microbial community changes under the pH stress.

2. Materials and Methods

2.1. Experimental set-up

The raw swine manure used in the experiments was sampled from a swine farm located in Kunshan, Jiangsu Province, China. Its characteristics are shown in Table 1. Before added into reactors, the raw manure was diluted by distilled water to 5.8 % of total solid concentration (TS) based on our preliminary experiments.

The batch experiments were carried out in seven 1000 ml ground flasks with working volume of 600 ml with initial pH of 3.0, 5.0, 9.0, 10.0, 11.0 and 12.0, labeled as R3, R5, R9, R10, R11 and R12, respectively by adding 5 M of sodium hydroxide (NaOH) or hydrochloric (HCl). The reactor at initial pH 7.0 (R7) was set as the control unit. After adjustment, each flask was immediately closed air-tightly by rubber caps with two holes left: one hole was used for sampling and the other for biogas collection. The flasks were placed in a thermostat shaker at 35±1ºC and 80 rpm for the fermentation which lasted for 20 d. Samples were collected every day for the first 9 days and thereafter every 2 days until the experiments stopped after 20 days.

2.2. Analytical methods

2.2.1. Chemical analysis

Samples from reactors were immediately centrifuged at 10000 rpm for 5 min and the supernatant was used for the detection of pH, soluble chemical oxygen demand (SCOD), volatile fatty acids (VFAs), total ammonium nitrogen (TAN), PO_4^3-P, carbohydrate and protein, while the sediment was collected for DNA extraction. The analyses of SCOD, PO_4^{3-}-P and TAN were conducted in accordance with Standard

Methods (APHA, 2005). Soluble protein was quantified by the Lowry-Folin method with bovine serum albumin (BSA) as standard (Lowry *et al*., 1951); and carbohydrate was determined using the phenol-sulfuric method with glucose as standard (Herbert *et al*., 1971).

Biogas production was monitored daily by water displacement method using a 500 ml graduated cylinder connected to each reactor. Methane (CH_4) content in the biogas produced and volatile fatty acids (VFAs, C2-C5) including Ac, Pr, n-Bu, i-Bu, n-Va and i-Va were determined by using gas chromatograph (7890A, Agilent, USA) fitted with HP-PLOT 80/100 Mesh (2 m×1/8" × 2.0 mm SS) packed column and TCD detection, and HP-FFAP (30 m × 0.25 mm × 0.25 μm) capillary column and FID detection, respectively.

The experimental data were expressed as the average of duplicate tests with deviation less than 4%.

2.2.2. Microbial community analysis

The DNA Isolating Kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA) was used to extract the genomic DNA from collected samples, and then the V3 region of 16S rRNA of the extracted DNA was amplified by polymerase chain reaction (PCR) as described previously (Wan *et al*., 2011) using universal eubacterial primers pair (8F, 5'-GAGAGTTTGATCCTGGCTCAG-3' with a GC clamp and 518R, 5'-ATTACCGCGGCTGCTGG-3'). The PCR products were separated by denaturing gradient gel electrophoresis (DGGE) using the Dcode™ universal mutation detection system (Bio-rad Laboratories, Hercules, CA, USA). Polyacrylamide gels with 30–60% range of vertical denaturing gradient were electrophoresed for 10 μl PCR product at

140 V and 60 °C for 8 hours and then stained by silver as described previously (Bassam *et al.*, 1991).

Dominant DGGE bands were excised and dissolved in 20 μl 1× TE，and 2 μl DNA solution was amplified by PCR under same conditions above. Then re-amplified PCR products were purified by agarose gel electrophoresis, ligated into vector pMD18 (Takara, Dalian, China), and cloned into E. coli DH5α. Positive clones were examined for ampicillin resistance by blue-white spot screening and sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing on the automated sequencer ABI3730. Nucleotide sequences were analyzed using the BLAST program in GenBank.

3. Results and Discussion

3.1. Swine manure hydrolysis

Figure 1 depicts the variation of SCOD under different initial pH's. At alkaline conditions (pH 10-12) the SCOD was higher than that of other pH's. Upon pH adjustment (day 0), the concentrations of SCOD were 17000 mg/L in R12, 7770 mg/L in R7, and only 6480 mg/L in R3. Restated, the COD was released very rapidly within 5 min after the pH was adjusted to 12. In the subsequent hydrolysis and acidification steps for 20 d, the SCOD concentration was increased by 62.4%, 80.5%, 89.3%, 34.1% and 25.3% in R3, R5, R10, R11 and R12, respectively. The SCOD in R7 and R9 were first increased rapidly by 49.8% and 30.3% within 4 days of testing, but were then decreased quickly accompanied with biogas production (data not shown), being attributable to the activities of methanogens. On the basis of SCOD release, culture pH at 10-12 was beneficial for SCOD production of swine manure, particularly being treated within very short period of time. The discussion on the effects of culture pH on SCOD production from sludge has been given by Kang *et al*. (2011).

3.2. *Swine manure acidification*

In R3 and R5 the VFAs concentrations were increased with time and peaked on day 11 and day 15, respectively (**Fig. 2**). In R10–R12 the VFA concentrations were increased linearly with time. At initial pH 7 and 9 the VFA followed a similar pattern as SCOD, first increased and then decreased in the testing. The highest concentration of total VFAs in each reactor was in the following order: R10 (13600 mg/L) > R11 (12000 mg/L) > R5 (10300 mg/L) > R12 (10000 mg/L) > R7 (8140 mg/L) > R9 (7720 mg/L) > R3 (7000 mg/L). The initial pH 10 or 11 was efficient for VFA production from swine manure with 5-8 times higher in VFA concentration than that from active sludge reported previously (Chen *et al*., 2007; Kang *et al*., 2011); however, although pH 12 could yield higher SCOD than pH 10 or 11, the corresponding VFAs production quantity was lower.

The distributions of yielded VFAs provide information on the involved metabolic pathways (Parawira *et al*., 2004). The Ac and Pr accounted for 51.8–71.2% of total VFAs amounts (**Table 2**). Concentrations of Ac were significantly increased at initial pH 10-12, particularly at pH 10. In R10 the Pr production also reached the maximum concentration of 3380 mg/L. Conversely, the concentrations of i-Bu and n-Bu were the highest in R5, indicating that butyrate-producing bacteria were adaptive to pH 5.

Among the six detected VFAs, HAc is the major precursor of methane (Lata *et al.*, 2002) and had economic value in chemical markets. As shown in **Fig. 3**, alkaline pH could not only enhance production of total VFAs, but also facilitate the Ac fermentation-type pathway.

3.3. Compositions of main organic carbons

The Ac, Pr and Bu can be formed directly from the fermentation of the soluble proteins, carbohydrates and lipids (Horiuchi *et al.*, 2002); while i/n-Va was mainly produced from proteins degradation (McInerney, 1988). In R7-R9 methanogens were active to degrade VFAs to methane (CH_4). Thus, the concentrations of soluble proteins, carbohydrates, lipids and the detected VFAs were a consequence of a balance between release/production and degradation (Chen *et al*., 2007). The concentration of lipid in swine manure was comparatively low (<1% of total COD), the major organic carbons in the present fermentation systems were proteins, carbohydrates, VFAs and methane.

Figure 4 depicts the compositions of the four major organic carbons before and after the 20-d fermentation. Except R7 and R9, none of the others displayed noticeable methane production, probably due to the inhibition of methanogens after pH adjustment. Interestingly, the accumulative methane production in R9 was 116.9 % higher (as COD) than that in R7, which was possibly caused by its higher initial concentration of VAFs (Figs. 2 and 4) and appropriate pH environment by VFAs neutralization.

Before fermentation, concentrations of soluble proteins and carbohydrates were increased with initial pH. The decrease in soluble proteins in acidic environment was probably due to the re-adsorption of the released soluble carbonaceous back to the solid matrix (Kang *et al.*, 2011). After 20 days of hydrolysis and acidification, concentrations of soluble proteins and carbohydrates were increased from 1020 mg/L and 1420 mg/L to 1810 mg/L and 1550 mg/L in R3, and were decreased from 6120 mg/L and 6220 mg/L to 5360 mg/L and 5470 mg/L in R12, respectively. The

concentrations of the soluble proteins and carbohydrates in R11 and R12 were higher than those in R10, likely owing to the inhibition of activities of acidogens at very basic environment. Initial pH 10 presents the optimal condition for VFA production from swine manure.

3.4. Ammonium release

Large amounts of ammonium from swine manure were released with hydrolysis and acidification (**Fig. 5**). Before fermentation, base dose had no effects on ammonium concentrations while acid dose significantly released the ammonium from the manure, which was attributable to quick deamination reactions between hydrochloric acid and amino acids. During fermentation, ammonium was gradually released by additional 300-400 mg/L, in accompany with the progress of the subsequent hydrolysis and acidification.

At the end of fermentation, the ammonium concentration was as follows: R10 (773 mg/L) > R11 (646 mg/L) > R12 (632 mg/L) > R5 (575 mg/L) > R7 (542 mg/L) > R9 (511 mg/L) > R3 (349 mg/L), correlating with the order noted for the highest concentration of VFAs, indicating that the released ammonium was principally yielded from acidification reaction. The biogas production can be inhibited at total ammonium of >1500 mg-COD/L and pH >7.4 (Van Velsen, 1979). The low ammonium levels noted for the present alkaline tests suggested that the released VFAs are readily useful for methanogens if the subsequent step is methanogenesis.

3.5. Microbial community structure

The DGGE molecular profiling of PCR amplified eubacterial was performed on swine manure before pH adjustment (original) and at the end of fermentation tests at

initial pH 3-12 (**Fig. 6**). A total of 18 major bands in the DGGE profiles were identified, whose closest relatives in the NCBI database were given in **Table 3**.

The DGGE fingerprints of all samples were significantly different. The bands 9, 10, 11 and 14 had high intensities in the initial control sample. The sequencing result showed that bands 9 and 14 were related to *Pseudomonas*, a facultative psychrophilic bacterium producing acids and hydrolytic enzymes for proteins (Salwan *et al.*, 2010; Yumoto *et al.*, 2001). The band 11 was highly homologous (98%) to *Clostridium sp.*, which could ferment organics to sugars, ethanol, acetate, lactate and hydrogen (Syutsubo *et al.*, 2005) as one of the most represented bacterial families in anaerobic digesters (Palatsi *et al.*, 2010). The bacterium corresponding to band 10 was detected in the samples of original manure and in R7–R12, which was belonging to *Corynebacterium aurimucosum*, a facultative anaerobe producing acids, could tolerant a wide range of environmental pH's. Restated, the original manure was enriched with *Pseudomonas* sp., *Clostridium* sp. and *Corynebacterium* sp.

After 20 d of hydrolysis and acidification, the structure of microbial community at different initial pH's has experienced significant shifts. In R7 and R9, a new band 5 became dominant, which was noted in a biogas-producing digester for co-fermentation of maize silage and bovine manure (Souidi *et al.*, 2007). The DGGE profile at pH 9 has an additional band 8 when compared with that at pH 7, which may be belonging to *Clostridium alkalicellum.* In fact, the strain *Clostridium alkalicellum* (band 8) was dominant at all alkaline tests, which is an obligatory, alkaliphilic, and halophilic bacterium, growing best at pH 9 and 0.15–0.3 M Na^+ (Zhilina *et al.*, 2005). The poor homology (86%) of band 8 also indicates the possibility of its belonging to a new taxon.

In R10 with the highest concentration of acid production, band 13 was the unique

bacteria present in the profile, relating to *Corynebacterium humireducens*, a halotolerant, alkaiphilic, humic acid-reducing bacterium with optimum conditions at pH 9 and 37 ºC (Wu *et al.*, 2011). The absence of band 13 at pH 11 and 12 suggests that the *Corynebacterium humireducens* has less tolerance on strong alkaline environment than that for band 8. The band 3 is presented at pH 11 and 12, which is relating to *Proteiniphilum acetatigenes* (Chen and Dong, 2005) and may be enriched owing to the increased soluble proteins in suspensions as substrates to grow. Differently, the *Ruminobacillus xylanolyticum* and *Uncultured prokaryote* corresponding to band 4 and band 6 respectively were present in at pH 12, both of which were found in the carbohydrate feeding digesters. The DGGE fingerprints hence revealed that the base adjustment led to significant microbial community shift with bacteria corresponding to band 8 and band 13 which play important role for high acidification efficiency of swine manure.

Compared with base adjustment, acid adjustment also led to marked microbial community shifts. In R5 the *Eubacterium* sp. (band 12) and *Butyricimonas* sp. (band 15) were noted, which were reported to produce high concentrations of ammonium (Attwood *et al.*, 1998) and n/i-Bu (Sakamoto *et al.*, 2009), respectively. This occurrence correlates with the high levels of ammonium (**Fig. 5**) and n/i-Bu (**Table 2**) produced at pH 5. Correlating with the high VFAs production efficiency, the community at pH 5 and pH 10 shared one common bacterium of band 18 (*Bacterium MNFS-9*), which might be another important bacterium for efficient acidification.

3.6. Implications to practice

As Zhang *et al.* (2012) presented, pH adjustment is effective to manipulate hydrolysis and acidification reactions for organic waste. In their work, intermittent pH

adjustment was applied to enhance hydrolysis and acidification reactions. Repeated pH adjustment is not practical in large-scale applications. In the present study, the initial pH was demonstrated to shift the microbial community in the suspension so as to affect hydrolysis and acidification reactions on swine manure. Restated, at pH 10 the strains *Clostridium alkalicellum* and *Corynebacterium humireducens* were enriched, likely contributing to the high Ac and Pr production from the manure. At pH 5, the strain *Butyricimonas* sp. was presented to produce high level of n/i-Bu. The collected manure is to be adjusted its pH once, then the rest of the reactions can be carried out with no further care.

In practice, if the bio-gas production is of concern, the manure at initial pH 7 or 9 is preferred for hydrolysis, acidification and methanogenesis. If the hydrolysis of swine manure is considered, the alkaline environment pH 10-12 is preferred. When the accumulation of acetate is the target, initial adjustment at pH 10 is recommended. Conversely if the butyrate production is preferred, pH 5 is the condition to be adjusted.

4. Conclusions

The effects of initial pH adjustment (pH 3-12) on hydrolysis and acidification of swine manure were studied. Alkaline environment (pH 10-12) enhanced SCOD and VFAs concentrations in suspensions, especially at pH 10 with which *Clostridium alkalicellum* and *Corynebacterium humireducens* were enriched, the VFA production peaked at 13600 mg-COD/L, 71.8% of which contributed by Ac and Pr. At pH 7-9 methane was produced from VFAs. In acidic environment ammonium was released in excess. At pH 5 the strain *Butyricimonas* sp. may be the dominant strain for enhanced n/i-Bu production.

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Table 1 Characteristic of the swine manure used in the experiment.

Parameter	**Unit**	**Value**
TS (total solids)	%	21.2±0.3
VS/TS (volatile solids/total solids)	%	83.2±0.1
pH	-	7.03±0.01
SCOD (soluble chemical oxygen demand)	mg/L	29800±800
Soluble proteins	mg/L	6000±90
Soluble carbohydrates	mg/L	5620±150
PO_4^{3-}-P	mg/L	205±13
TAN (Total ammonium nitrogen)	mg/L	2690±45

Table 2 VFA spectrums of the initial control sample (pH 7 on day 0) and fermentative samples of different initial pH's on the corresponding day of highest concentration of total VFAs (Unit: mg-COD/L).

pH	**Time**	**Ac**	**Pr**	**i-Bu**	**n-Bu**	**i-Va**	**n-Va**
7	Day 0	2230	1200	394	831	577	389
3	Day 11	2200	1420	532	1260	989	593
5	Day 15	3480	1970	920	1610	1430	867
7	Day 3	2750	2390	591	1070	1010	334
9	Day 3	2770	2320	617	850	1110	292
10	Day 20	6330	3380	849	1360	1340	375
11	Day 20	5650	1830	680	1190	1320	299
12	Day 20	4940	1680	687	956	1440	327

Table 3 Sequence analysis of bands excised from DGGE gel in Fig 6.

Band ID	**Closest relative (NCBI database)**	**Accession number**	**Similarity (%)**	**Taxonomic group**
1	*Uncultured Prevotellaceae bacterium clone sz-264*	JQ279007.1	99%	*Bacteroidetes*
2	*Clostridium sp. 6-44*	AB596885.1	90%	*Firmicutes*
3	*Proteiniphilum acetatigenes strain TB107*	NR043154.1	92%	*Bacteroidetes*
4	*Ruminobacillus xylanolyticum*	DQ178248.1	94%	*Firmicutes*
5	*Uncultured bacterium clone ATB-KS-1507*	EF686980.1	99%	*Bacteria*
6	*Uncultured prokaryote clone*	HQ154873.1	99%	*Prokaryote*
7	*Clostridium mayombei train DSM6539T*	FR733682.1	95%	*Firmicutes*
8	*Clostridium alkalicellum*	AY959944.2	86%	*Firmicutes*
9	*Pseudomonas antarctica strain KJPB54*	FM213380.2	99%	*Proteobacteria*
10	*Corynebacterium aurimucosum strain NRRL B-24143*	AY536427.1	92%	*Actinobacteria*
11	*Clostridium sp. A7-9*	AB238882.1	98%	*Firmicutes*
12	*Eubacterium sp. C2*	AF044945.1	94%	*Firmicutes*
13	*Corynebacterium humireducens NBRC 106098 strain MFC-5 1*	GQ421281.1	99%	*Actinobacteria*
14	*Pseudomonas psychrophila strain E-3*	NR028619.1	99%	*Proteobacteria*
15	*Butyricimonas sp. 214-4*	AB739696.1	84%	*Bacteroidetes*
16	*Bacterium L4M2 1-9*	AY862593.1	86%	*Bacteria*
17	*Sphaerochaeta sp. GLS2*	JN944166.1	98%	*Spirochaetes*
18	*Bacterium MNFS-9*	HQ823674.1	99%	*Bacteria*

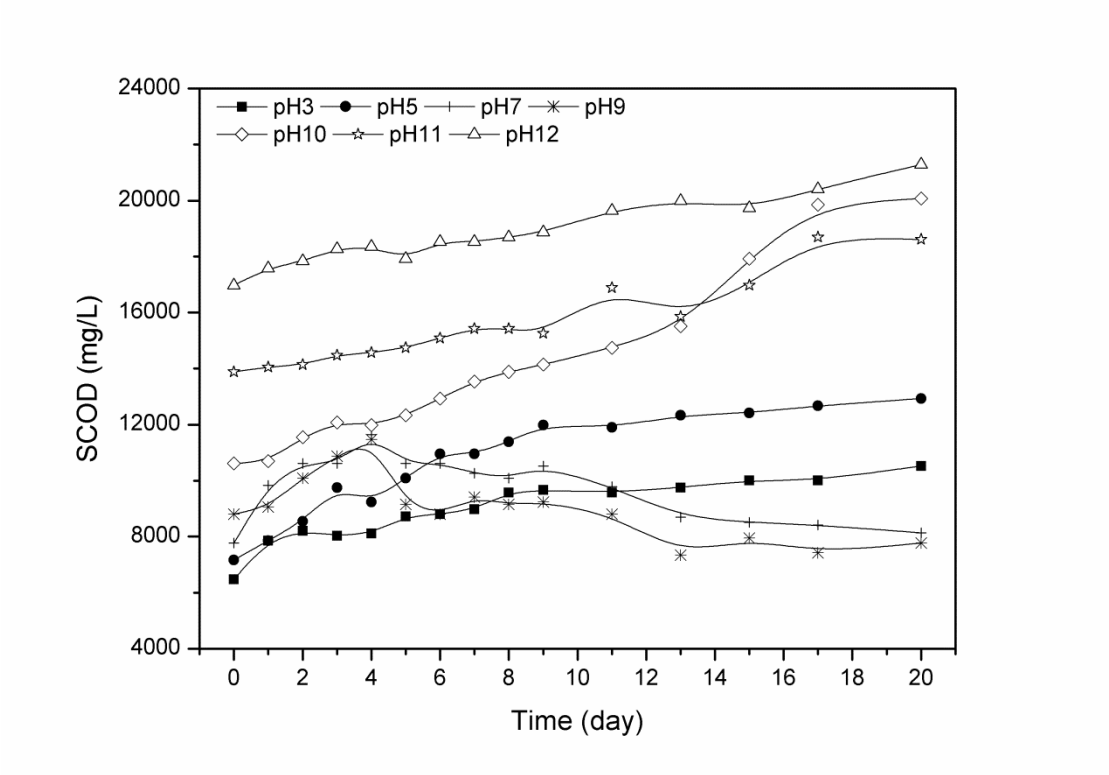

Figure 1. Release of SCOD under different initial pH conditions during hydrolysis and acidification process of swine manure.

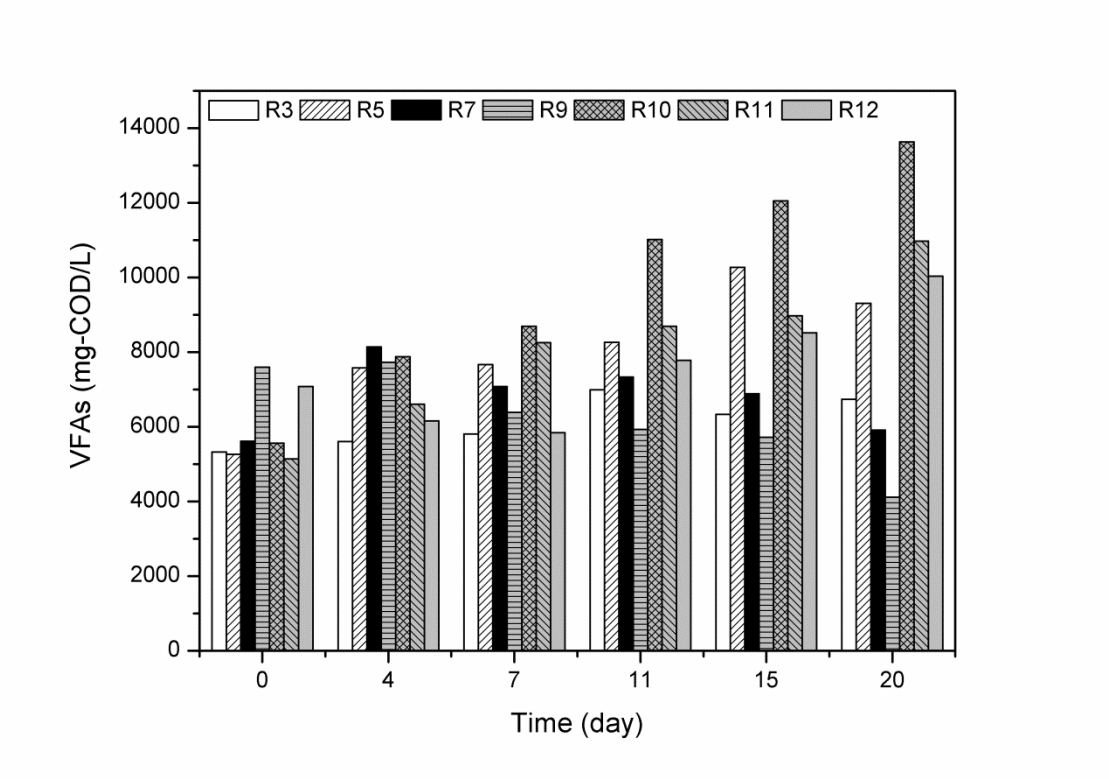

Figure 2. Variation of VFAs under different initial pH conditions during hydrolysis and acidification process of swine manure.

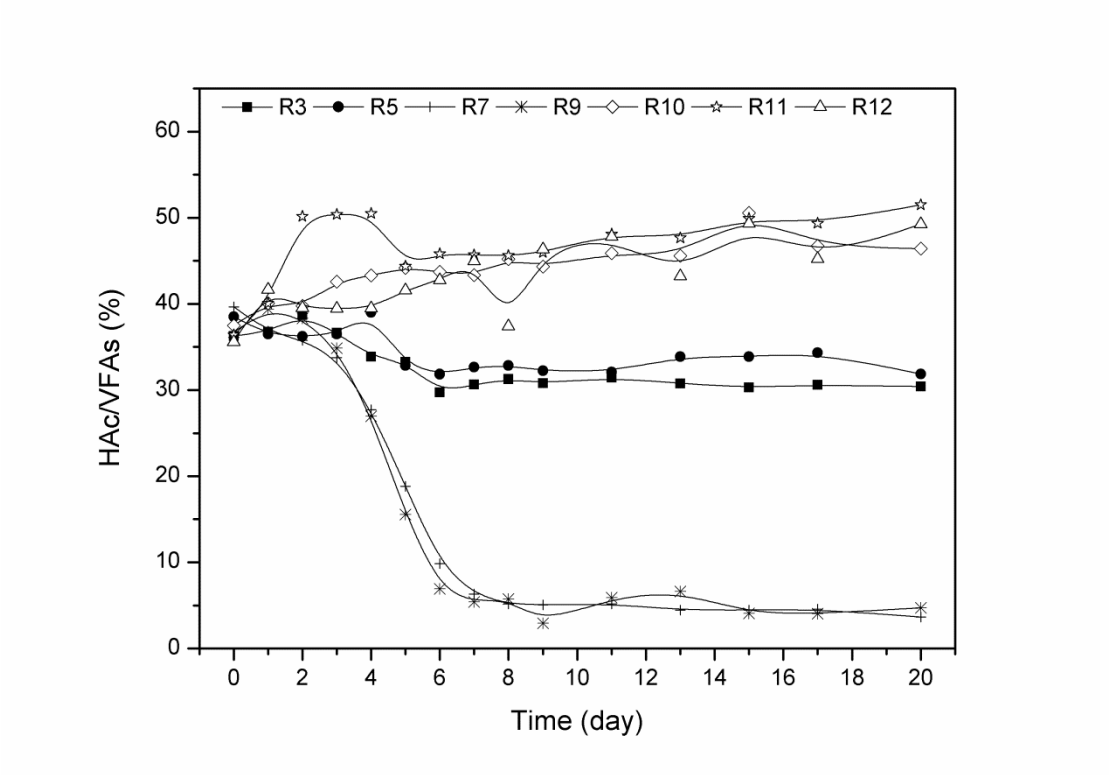

Figure 3 Variation of HAc/VFAs ratios under different initial pH conditions during hydrolysis and acidification process of swine manure.

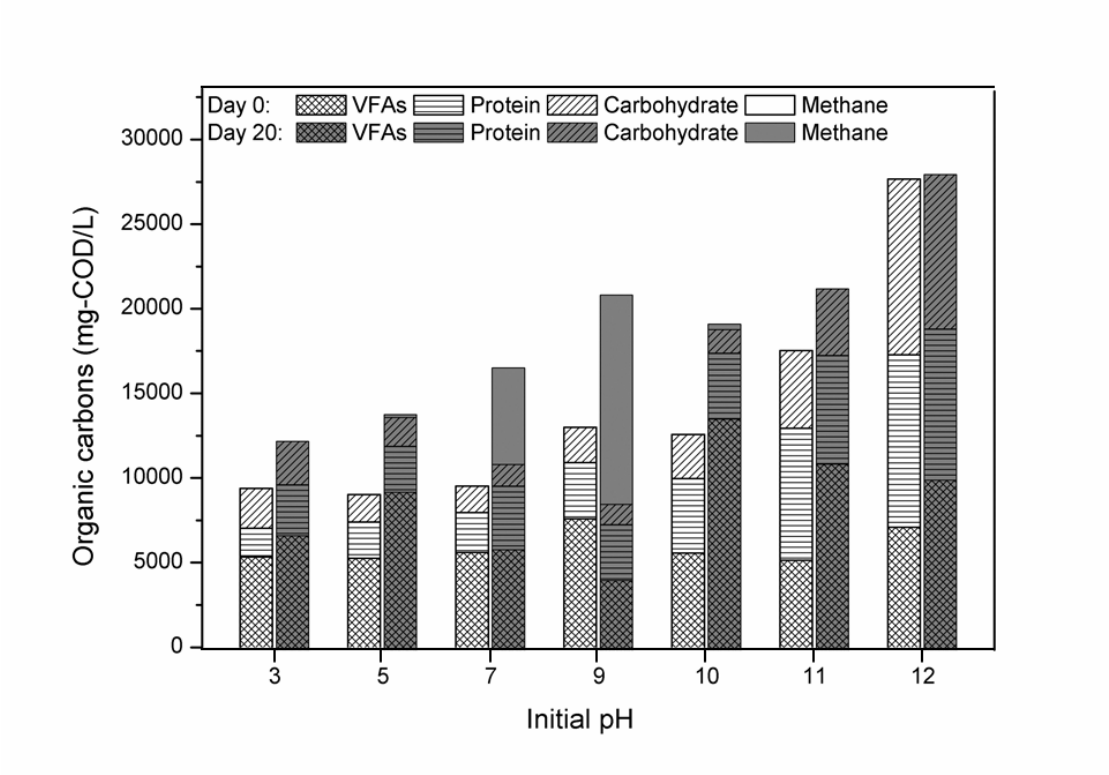

Figure 4 Composition of main organic carbons (soluble VFAs, protein, carbohydrate and accumulative methane) under different initial pH conditions on day 0 and day 20.

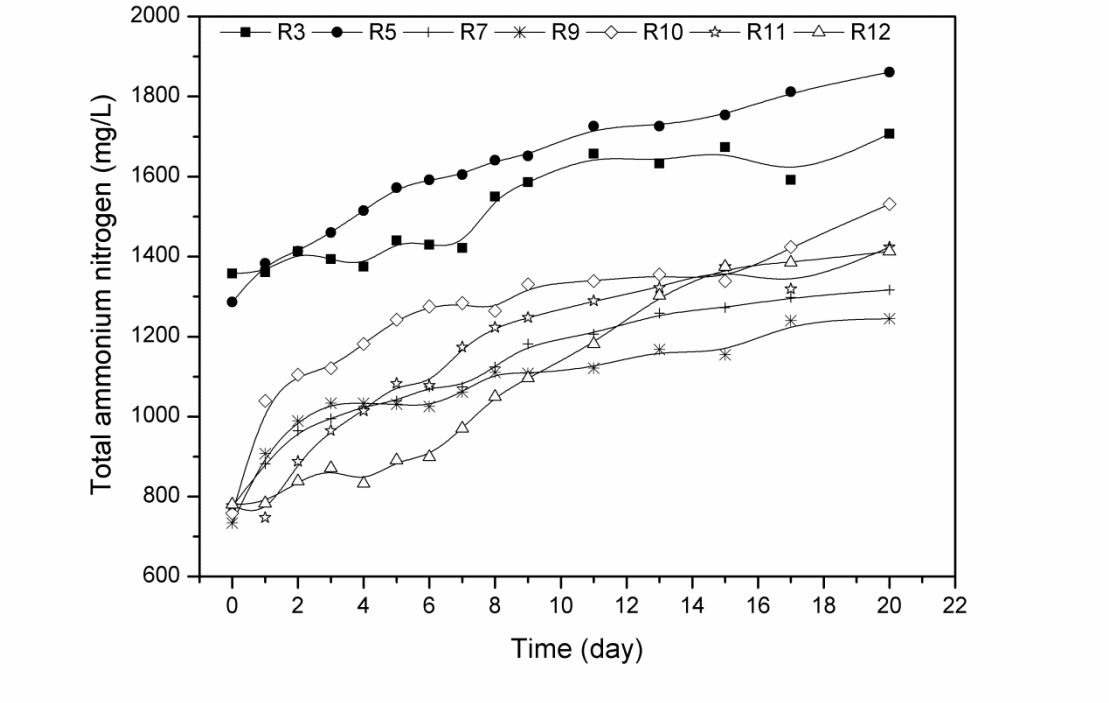

Figure 5. Release of total ammonium nitrogen (TAN) under different initial pH conditions during hydrolysis and acidification process of swine manure.

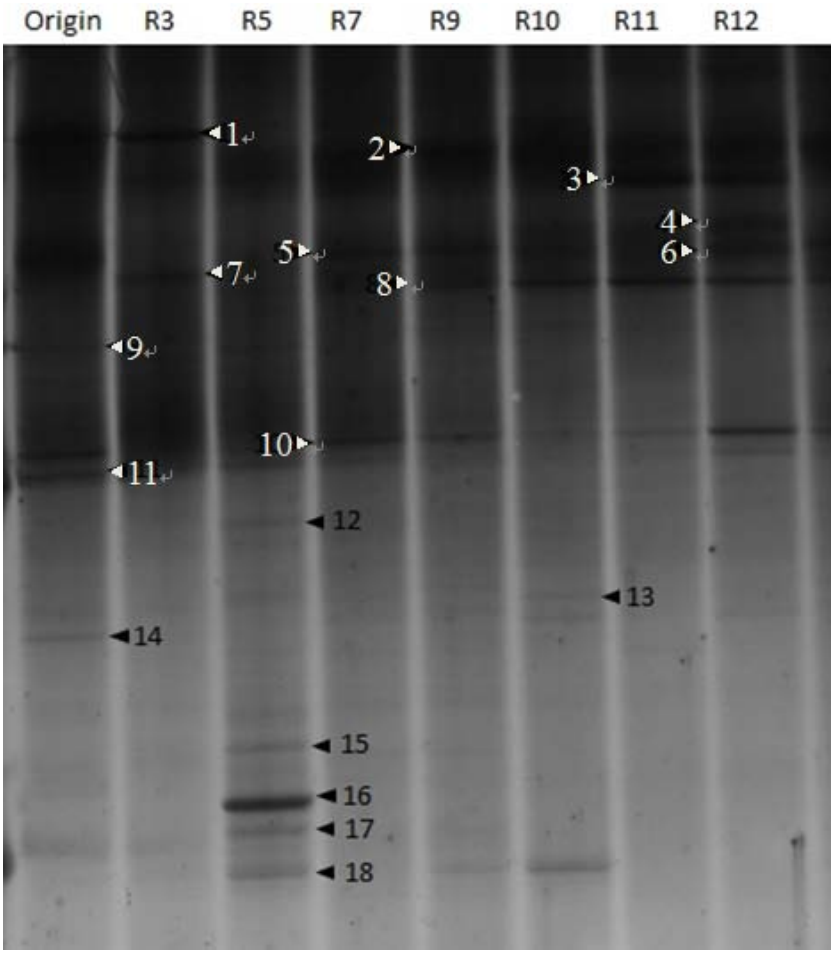

Figure 6. DGGE profiles of PCR-amplified 16S rDNA fragments from the microbial community of the initial control sample (Original) and the final samples in each reactor (R3-R12).