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Volatile fatty acid production from vegetable waste: Effect of organic components in mixed substrates

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Abstract
To investigate the effect of organic components in substrate on the performance of volatile fatty acids (VFAs) production, four vegetable wastes (carrot, potato peel, celery and Chinese cabbage) were mixed in different proportions and used as substrates for anaerobic fermentation. The result showed that the total VFA yield of the substrate with 50% potato peels was the highest (17888.24 mg COD/L). The high proportion of carrot and Chinese cabbage in the substrate led to the inhibition of hydrolysis and low VFA production. The decomposition of proteins in potato peels had a buffer effect on the reduction of pH and was conducive to VFA production. The metabolic pathway with carrot and Chinese cabbage as the main substrate was ethanol-acetic acid type, while that with potato peels and celery as the main substrate was butyric acid type. Lactobacillus, Psedumonas and Clostridium_sensu_stricto were the main functional microbes in the reactor with potato peels as the main substrate.

Keywords: *Anaerobic fermentation; Vegetable waste; Organic components; VFA production; Metabolic pathway*

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I. Introduction

Nowadays, in order to reduce the burning of fossil energy and properly dispose of organic municipal, agricultural and agro-industrial waste [1],using organic waste to produce energy has become a hot topic. Globally, 1.3 billion tons of agricultural products are wasted every year in production, processing, transportation, storage, and distribution process, accounting for one-third of total global food production [2]. There are 25% of the vegetables wasted in the production chain [3]. Normally, ordinary garbage is treated by landfill, incineration, crushing and direct discharge. However, due to the high water content (>80%) and high organic content, vegetable wastes (VWs)are easy to be degraded by microorganisms, which has a serious negative impact on the landfill and incineration system, such as a large amount of leachate polluting groundwater, unstable combustion producing toxic gas, etc. [4].

In addition to traditional waste disposal methods, anaerobic fermentation is a technology that is compatible with a variety of waste products biodegradation[5]. Carbon-rich VWs areideal substrates for anaerobic fermentation. They contain a large amount of organic matter, such as cellulose, hemicellulose, lignin, pectin, starch and various minor components (protein, fatty acid)[1], which can be converted into value-added fuels and chemicals (VFAs) through anaerobic fermentation.VFAs are important chemical raw materials, composed of two to six carbon atoms, such as acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, caproic acid[6]. They can be used as the base materials for bioenergy production and bioplastics production, and have been used in many fields such as medical, leather, textile, plastics and energy industries [7, 8]. Moreover, short-chain VFAs can be converted into long-chain fatty acids with higher value under certain conditions [9]. Therefore, it is a feasible and promising method to treat vegetable waste by anaerobic fermentation technology to produce VFAs.

The previous studies suggested that the vegetable species had significant effects on anaerobic metabolism typeand the production and distribution of fatty acids [10]. Zhang et al. [11] used four vegetables

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(potato peel, carrot, celery and Chinese cabbage) as substrates for anaerobic fermentation, and found that the metabolism pathway of the four vegetable wastes were propionic acid type, butyric acid type, mixed acid type and ethanol-acetate type according to the distribution of VFAs, respectively. Kumar et al.[12] used potato, tomato, cabbage, carrots and other vegetable wastes as mixed substrate in anaerobic fermentation, and found that acetic acid accounted for 54% of the totalVFAs (TVFAs). In the same year, Ravi et al.[13] used a mixture of carrots, celery, cabbage and potatoesas substrates for anaerobic fermentation and reported that a increase in acetic acid production of 15% buta decrease in valeric acid production of 10% compared to former in this system. Although mixed substrates have usually employed for VFA component analysis, the research focused on the effects of specific VWs mixture composition on the characteristics of VFA production by anaerobic fermentation is still limited.

A global production of potato is around 368 millions tones[14]. Potatoes are widely used in the processing of French fries, chips, puree and other foods. Annually, these industries produce 70 to 140 thousands tons of potato peels worldwide [15]. The starch content in potato peels is as high as 50% [14], which is an ideal substrate for anaerobic fermentation. In the juice industry, thousands of tons of carrot pomace are produced after the juice extraction [16,17]. And carrots pomace was rich in insoluble Fiber-rich fractions (50.1-67.4 g/100g) [18], which can be used as a representative vegetable with high content of polysaccharides. Zhou et al. [19] reported that the total annual production of Chinese cabbage is approximately 30 millions tones and up to 30% of the total production is discarded as waste. The fiber content of Chinese cabbage waste accounts for 26.5% of dry matter [20]. Celery, one of the most abundant vegetables in the world, is cultivated worldwide and utilized in food and cosmetic industries [21]. It contains considerable amounts of cellulose, hemicellulose and lignin, and therefore is relatively difficult to degrade[22].

To study the effect of substrate composition on the performance of the VFA production, four representative vegetables, i.e. potato peels (rich in starch), carrots (rich in soluble polysaccharides), Chinese cabbage and celery (rich in cellulose) were selected as feedstocks. Batch anaerobic fermentation was conducted with the above four VWs mixed in different proportions. The changes of the system pH value, SCOD concentration, VFA/SCOD ratio, VFA production and distributionduring the fermentation process were analyzed. By exploring the effects of specific VWs composition on the anaerobic fermentation characteristics, this study provided meaningful information for the development and utilization of VWs anaerobic fermentation to produce VFAs.

II. Materials and Methods

2.1 Inoculum and substrate

The seed sludge used in this study was taken from a local biogas production plant (Zibo, Shandong province, China) and stored at 4℃. The digester was operated at mesophilic condition and fed with bovine manure. Prior to use, the inoculum was cultured at 37℃ for 3 days. Potato peels were collected from the canteen of Shandong University of Technology. Carrots waste, Chinese cabbage waste and celery waste were collected from a local vegetable market. The VWs were minced by a high speed blender, respectively, and then mixed in the designed proportions. The characteristics of the VWs and the seed sludge were shown in Table 1.

2.2 Batch digestion design

Batch digestions were conducted in four 5L continuous stirred tank reactors. The VWs pulp were mixed in the proportions shown in Table 2 based on volatile solid (VS) and the mixture were labeled as S1, S2, S3 and S4, respectively. The VWs pulp mixture and the seed sludge was mixed in the ratio of 9:1 (based on VS). Tap water was used to regulate the total solid content (TS) to 4.5%. Before running, the reactors were flushed with nitrogen for 3 min to eliminate oxygen. The reactors were maintaind at 37± 0.5°C by the circulating water in the jacket and run for 144 h with a stirring speed of 300 rpm. During the fermentation process, the fermentation broth was withdrawn from each of the 4 reactors every 12 h for analysis. All the tests were carried out in triplicate.

2.3 Analytical methods

The pH of the fermentation broth was measured and documented online in real-time via a pH meter connected to the reactor. The analyses of TS and VS were conducted in accordance with standard methods [23]. Soluble chemical oxygen demand (SCOD) and total chemical oxygen demand (TCOD) were measured using a COD analyzer (6B-3000A, Shengaohua Co., China) [24]. The determination of VFAs were used a gas chromatograph (GC-6890, Agilent) equipped with a Flame Ionization Detector (FID) and a Nukol free fatty acidphase fused silica capillary column (DB-FFAP, 30 m×0.53 mm×1.0 μm, Agilent).The detail protocol was described in Zhang et al. [11].The concentrations of VFAs were covered to COD using the conversion factor that represented the mass of oxygen being consumed by 1 g organics when it was completely oxidized to CO_2 and H_2O [25]. The corresponding conversion factors for ethanol, acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, and hexanoic acid were 2.09, 1.07, 1.51, 1.81, 1.81, 2.04 and 2.21, respectively.

2.4. Microbial community analysis

Microbial communities in the inoculum (labeled as In) and the broth of S2 after 108 h fermentation (labeled as S2) were analyzed via high-throughput sequencing by Sangon Biotech Co. Ltd. (Shanghai, China). DNA was extracted using the OMEGA kit E.Z.N.ATM Mag-Bind Soil DNA Kit. After precise quantification of genomic DNA using the Qubit 3.0 DNA Detection Kit to determine the amount of DNA that should be added to the PCR reaction. The primer pairs were 341 F (5′ - CCTACACGACGCTCTTCCGATCTN-3′) and 805 R (5′-GACTGGAGTTCCTTGGCACCCGAGAATTCCA -3′). Sequencing was performed on the Illumina MiSeq high-throughput sequencing platform.

III. Results and discussion

3.1 Total VFA yield

The composition of organic matter in the substrate is an important factor affecting the yield and distribution of VFAs in anaerobic fermentation[24]. The TVFA yields of the VWs mixed at different proportions during fermentation were shown in Fig. 1.The initial TVFA concentration of S1 group was higher than that of other groups in the initial 12 h, but it increased slowly in the subsequent fermentation process.Carrot waste was the main substrate of S1. As shown in Table 2, the soluble sugar in carrots were 8.39% [26], which can be degraded into glucose for rapid utilization by acidogenic microorganism [5, 27]. Soluble matters played positive roles on the initial accumulation of VFAs, which may also be one of the reasons for the rapid production of VFAs in group S1. The accumulation of VFAs resulted in a dramatic decrease in pH to 4.0 within 72 h (Fig. 4). The lower pH suppressed the activities of microorganism [25], resulting in no significant increase in SCOD release and VFA production (Fig. 2) in the late fermentation stage. With Chinese cabbage waste as the main substrate, the VFA yield of S4 group increased rapidly in the initial stage of fermentation, indicating that Chinese cabbage contains soluble substances that are easily degraded by hydrolyzing microorganisms, and used by acidogenic bacteria. However, there was no significant increase in VFA yield from 24 hours to the end of fermentation.Similar to S1, the pH of S4 decreased rapidly in the initial stage of fermentation, which may also be the reason for its low VFA yield.

The cumulative trends of VFA yield in S2 and S3 groups were similar, which increased slowly in the early stage of fermentation, and then suddenly increased rapidly (Fig. 1). However, the rapid increase of VFA production in S2 group was 24 hours earlier than that in S3 group. The fermentation substrates of S2 group and S3 group were mainly potato peels and celery waste, respectively. During fermentation process, different organic components, from simple to complex, were degraded and consumed sequentially [28]. Cellulose is more difficult todegrade than starch, and requires additional hydrolysis steps to release fermentable sugars for acidified bacterial metabolism [28]. The main substrate in S3 was celery waste, so the content of cellulose was high and the soluble carbohydrate was low, which led to the slow accumulation of VFA production in the initial stage of fermentation. With the hydrolysis of cellulose and other substances, the substrates that can be used by acidified bacteria gradually increase [29]. Subsequently, the concentration of VFAs quickly increased and reached 17732.52 mg COD/L at 144 h.However, using only celery as fermentation substrate, the VFA yield remained low within 117 hours and reached about 12000 mg COD/L at 150 hours[11]. This result suggested that the incorporation of readily degradable components in cellulose-rich materials promoted the development of the acetate pathway, which increased VFA yield [28].

3.2 Release of SCOD

The increase of SCOD concentration reflected the conversion of solid substrate to soluble organics in the hydrolysis step of anaerobic digestion [30]. Therefore, the degree of SCOD release was usually used to evaluate the performance of substrate hydrolysis.Starch is more easily degraded by hydrolyzing microbes than cellulosic materials [28]. Using potato peels as the only substrate for fermentation, the release rate of SCOD was faster than that of celery as substrate [11, 31]. As shown in Table 2, potato peels accounted for 50% of the fermentation substrate in S2, indicating that starch was the main component. However, the SCOD concentration of S2 was lower than that of S3, only 9501.5 mg/L in the first 36 h (Fig. 2). Chau et al. [18] reported that the insoluble fiber-rich fractions of carrots have high glucose-adsorption capacity and amylase-inhibition activity. Thus, the addition of easily degradable carrots to the starch-rich potato peels can significantly reduce the release of SCOD in the prophase of fermentation. The SCOD concentration of S2 increased rapidly after 36 h (Fig. 2), which might because the inhibition of amylase decreased with the degradation of carrot polysaccharides and the starch in the substrate began to hydrolyze rapidly.

Celery waste accounted for 50% in S3, so its cellulose content was higher than that of the other three groups. Refractory cellulose was not readily utilized by hydrolyzing microorganisms, resulting in slow SCOD accumulation during the early stage of fermentation. After 72 h, the cellulose in the substrate began to be used by hydrolyzing microorganisms, and the SCOD of S3 gradually increased, reaching 21610.35 mg/L at 144 h.

Although the substrate composition of S4 was mainly cellulose, similar to that of S3, the concentration of SCOD did not increase rapidly, but remained at a low level.Fang et al. [32] reported that the hydrolysis of lignocellulosic biomass were inhibited due to lower activities of cellulolytic bacteria at low pH. As shown in Fig. 3, the pH value of S3 increased slightly from the middle fermentation period, while the pH of S4 remained low. This might explain the considerably lower release of SCOD from S4 than from S3 after 72 h.

3.3 Changes of pH during anaerobic fermentation

The pH value of the fermentation environment not only affects the community structure of microorganisms, but also affects the absorption of nutrients by microorganisms[25].The optimum pH for the growth and metabolism of acid-producing bacteria is in the range of 5.2 to 6.5 [33]. In this study, the initial pH value of each group was about 6.5, which was favorable for the growth and metabolism of acidogenic bacteria. But the pH changes of the four experimental groups were quite different during the fermentation process (Fig. 3).

The pH value of S1 group decreased rapidly to below 4.5 within 24 hours. The excessively low pH inhibited the activity of microorganisms, resulting in lower VFA yield and SCOD concentration during the fermentation process. In fact, in low pH fermentation broth, VFAs existed in their undissociated form. This situation made VFAs more liposoluble, thereby increasing their ability to diffuse in the reaction medium and to penetrate into the bacterial cells. This would cause the pH value of the microbial cells to decrease, impairing the cell viability of the microorganism and consequently, and affect the production of VFAs [34]. The fermentation in a low performant steady state was more inclined to produce acetic acid [35], which consistent with the result in Fig. 4. From 36 h, the pH of S2 gradually increased, and the VFA yield and SCOD concentration also increased rapidly. This may be due to the delayed decomposition of proteins in fermentation substrates, especially in potato peels, releasing ammonia nitrogen, which has a buffer effect on the reduction of pH [11]. The moderate increase of pH relieved the inhibition to hydrolysis microorganisms and accelerated hydrolysis and acidification. Zhang et al. [11] reported that using Chinese cabbage as the only fermentation substrate, serious acid inhibition occurred and the VFA yield was only about 4000 mg COD/L. In the fermentation process, the lowest pH of S4 group with Chinese cabbage as the main substrate was 4.42, indicating that the addition of potato skin can buffer the sharp decline of pH in anaerobic fermentation of Chinese cabbage and the addition of carbon- rich substrate benefited the production of VFAs (7859.01 mg COD/L).

3.4 Effects of vegetable mixing ratio on the distribution of VFAs

The composition of anaerobic fermentation products has a greater impact on theirapplied field. The characteristics of the substrate are one of the main factors affecting the type of acid production (specific acid products) in the system [36, 37]. The variation of ethanol and individual VFA concentration during fermentation were determined. As shown in Fig. 4, the distribution of VFA products was significantly different due to the different mixing proportions of vegetable waste. The acid-producing type of S1 and S4 was ethanol-acetic acid type. The yield of acetic acid was higher than that of ethanol, accounting for 41.64% and 48.63% of the total products, respectively (Fig. 5). Meanwhile, the yield of butyric acid was very low and showed an obvious upward trend from 96h. However, the anaerobic fermentation with carrot as the only raw material was butyric acid type, and the fermentation products were mainly acetic acid and butyric acid, and the proportion of butyric acid reaches 54.00% of TVFA[11]. The reason may be that the addition of potato peels increased the starch content in the system, and glucose was generated with the degradation of starch. The acidogenic microorganisms were more likely to generate butyric acid by using glucose [28]. This was consistent with the result of Ma et al.[10] that the addition of starch delayed the production of butyric acid and decreased its proportion. The fermentation with Chinese cabbage as the only raw material was also ethanol-acetic acid type, but different from S4, the concentration of ethanol was considerably higher than that of acetic acid [11]. This result suggested that the addition of soluble sugars to cellulose facilitated the acetic acid pathway [28].

The concentration of butyric acid of S2 was 11600.24 mgCOD/L, accounting for 61.14%, which was a butyric acid type fermentation[38]. In addition to butyric acid, the main products of S2 also included acetic acid and propionic acid. However, Zhang et al. [11] found that the fermentation type of potato peels was a propionate-type metabolic pathway. The difference of substrate composition led to the uneven growth of different acid producing bacterial groups in the fermentation system, while the difference of microbial community structure led to the difference of fermentation products [39]. Butyric acid was also one of the main products of S3, and the maximum concentration was 8766.41 mg COD/L, accounting for 45.88%.

3.5 Diversity of the microbial community

In addition to the organic composition of the substrate, the microbial community structure is also an important factor affecting the fermentation products. Liang et al. [40] reported that the acetic acid and propionic acid were produced by PrevotellaandBacteroides, while the butyric acid and valeric acid were produced by

Ruminococcaceae and Lachnospiraceae. To further understand the relationship between substrate composition degradation and microbial communities, the classification and changes of microbes in the inoculum and the broth of S2 after 108h fermentation were analyzed. The abundance and diversity of biological communities can be reflected by diversity analysis of single samples (Alpha-diversity) in community ecology (Table 3). Ace and OTUswere applied to estimate the community distribution abundance. The diversity of the sample communities can be reflected by Shannon index and Simpson index [7]. As can be seen from Table 3, the Coverage values of the inoculum and the fermentation broth of S2 both were 1, indicating that the sequences in the samples were completely covered. The Ace values of In and S2 were quite different, and their values showed the same trend as OTUs. The microbial abundance of the fermentation broth was reduced compared to the inoculum, and more aerobic bacteria were inhibited due to the acidic and anaerobic conditions, which reduced the number of heterobacteria [41]. From Fig. 6A, it can be seen that almost all the bacteria of Chloroflexi disappeared.However, higher shannon index and lower simpson index resulted in higher species diversity in the fermentation broth. It may be that the natural microorganisms that come with the waste vegetables were enriched during the fermentation process, resulting in a more uniform distribution of microorganisms in the fermentation system.

As shown in Fig. 6,the relative abundance of microbial communities inthe inoculum and S2 are significantly different at both phylum level and genus level. The dominant bacteria in the inoculum at the phylum level were Chloroflexi and Firmicutes, with relative abundances of 47.0% and 36.0%, respectively.During the fermentation process, Firmicutes,Proteobacteria, and Actinobacteria were obviously enriched. Firmicutes contains diverse fiber degrading and acidogenic bacteria, which can produce acetic acid, propionic acid and butyric acid in the process of anaerobic fermentation [1]. The high content of carbohydrates in the raw material allowed the fermentation system to contain a high relative abundance of Firmicutes, playing an important role in the conversion of organic matter into VFA [42]. At the genus level, the dominant bacteria in S2 were Lactobacillus and Clostridium_sensu_stricto, both of which belong to Firmicutes. Lactobacillus can produce lactic acid and ethanol via the Phosphoketolase (PK) pathway, after which the accumulation of lactic acid lead to a rapid decrease in pH and thus inhibit the dissolution of the substrate [8, 35]. However, the relative abundance of Psedumonas from Proteobacteria increased from 0.01% to 9.10%.Pseudomonas could use lactic acid and glucose to produce acetate and butyric acid, thus neutralizing the pH depression caused by lactic acid accumulation and resulting in a steady increase in VFA production during the late fermentation [43]. As the fermentation progresses, it was reasonable to guess that the interaction of Lactobacillus and Psedumonas favored the production of butyric acid in the system. As a functional microbe that can produce butyric acid by glycolysis, Clostridium_sensu_stricto maintained a high relative abundance (23.06%-19.03%) throughout the fermentation process [44]. Actinobacteria was one of the most dominant hydrolyticbacteria in anaerobic fermentation,which was beneficial to the degradation of carbohydrates [19, 45, 46]. Since the relative abundance of Actinobacteria in S2 increased from 0.61% to 19.32%, the hydrolysis ability of the substrate was enhanced, so the higher level of SCOD concentration in S2 at 108h could be explained (Fig. 2). The relative abundance of Chloroflexi was reduced to 0.07% after fermentation. Chloroflexi showed carbohydrate consuming activity only under aerobic conditions, and the disappearance of this bacterium was a common phenomenon in anaerobic fermentation [47,48].

IV. Conclusions

Four representative vegetable wastes (carrot, potato peel, celery and Chinese cabbage) were mixed in different proportions and used as substrates for anaerobic fermentation. The mixing proportion of vegetable waste had a significant effect on the yield and distribution of fermentation products. A high proportion of refractory cellulose and starch in the substrate led to high butyric acid production. Using carrots or Chinese cabbage as the main substrates resulted in serious acid inhibition and low VFA yield. The incorporation of readily degradable components in cellulose-rich materials promoted the release of SCOD and increased the yield of VFAs. The addition of carrot waste to the starch-rich potato peels inhibited the release of SCOD in the prophase of fermentation. The metabolic type of anaerobic fermentation was closely related to the composition of vegetable waste. The metabolic pathway with carrot and Chinese cabbage as the main substrate was ethanol-acetic acid type, and the metabolic pathway with potato peels and celery as the main substrate was butyric acid type. Lactobacillus, Psedumonas and Clostridium_sensu_stricto played key roles in the butyrate-type metabolic pathways with potato peels as the main substrate.

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Figure Captions

Fig. 1. Variation of total VFA (S1: 50% carrot; S2: 50% potato peel; S3: 50% celery; S4: 50% Chinese cabbage).
Fig. 2. Changes of SCOD concentration.
Fig. 3. Changes of pH value of each group during anaerobic fermentation.
Fig. 4. Changes of ethanol and individual VFA concentration during fermentation.
Fig. 5. Distribution of ethanol and individual VFAs in the products.
Fig. 6 Relative abundance of dominant species in bacterial community structure (In and S2): at the phylum (A); at the genus (B).

Table captions

Table 1 Characteristics of the VWs and inoculum.
Table 2 Proportions of the VWs pulp.
Table 3 Comparison of the diversity of bacterial community in different samples

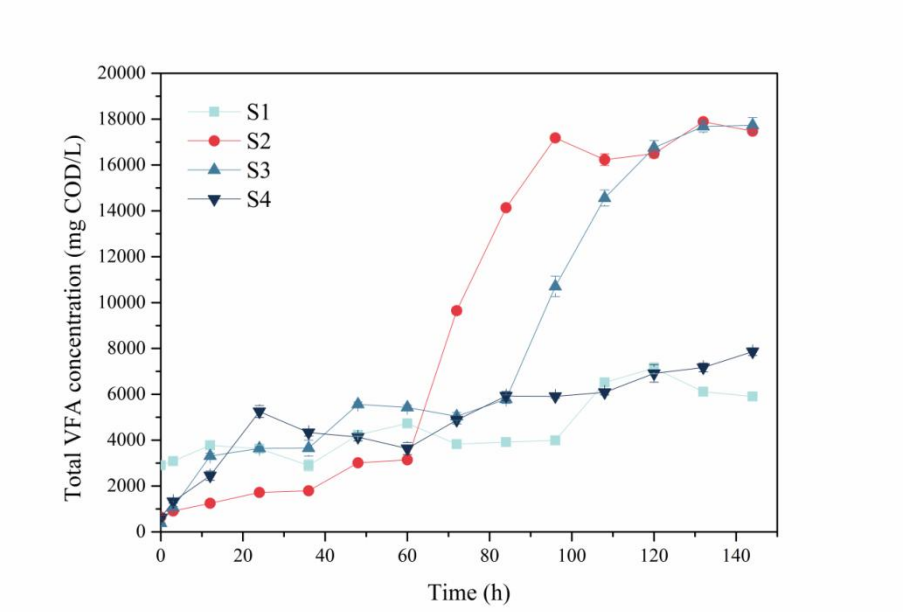

Fig. 1. Variation of total VFA yield (S1: 50% carrot; S2: 50% potato peel; S3: 50% celery; S4: 50% Chinese cabbage).

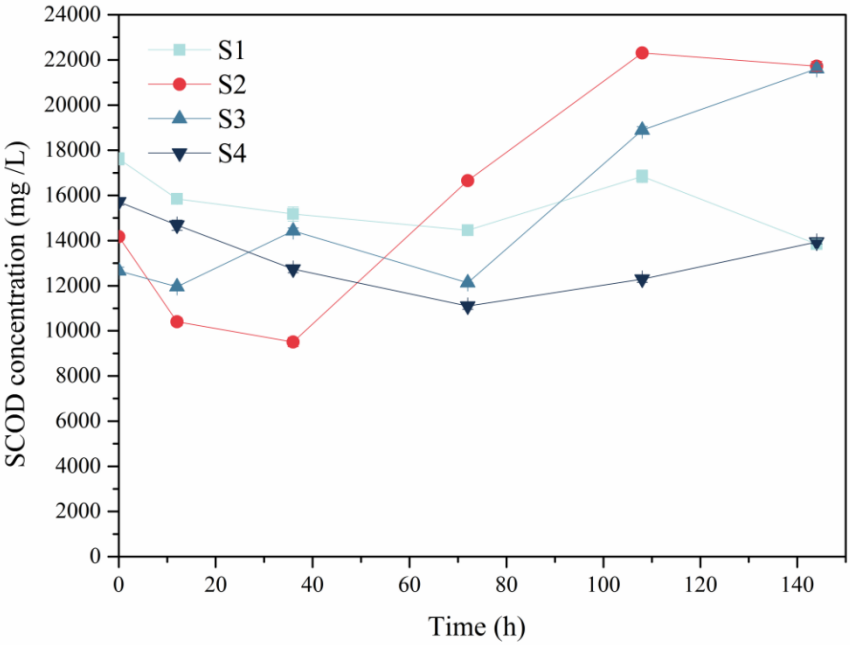

Fig. 2. Changes of SCOD concentration.

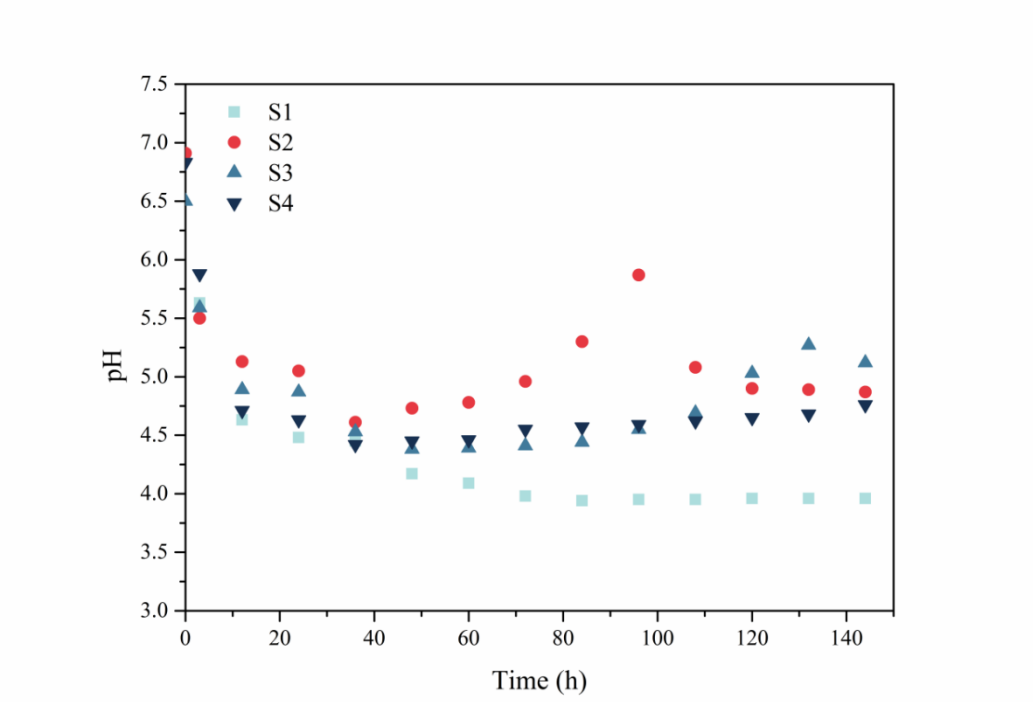

Fig. 3. Changes of pH value of each group during anaerobic fermentation.

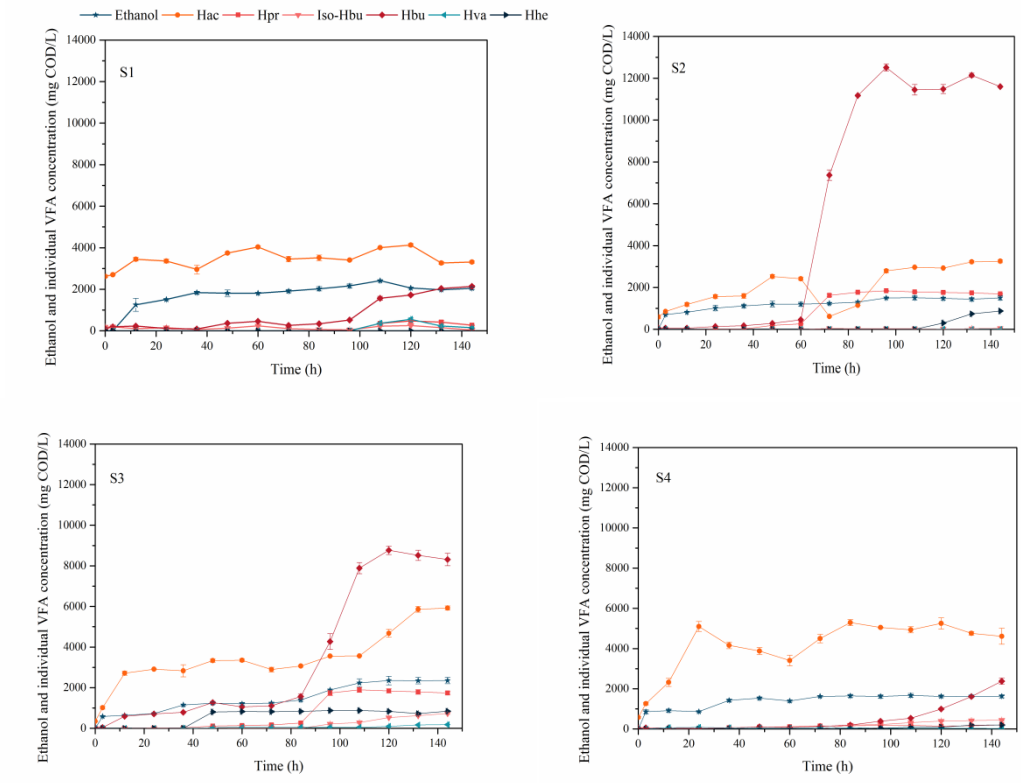

Fig. 4. Changes of ethanol and individual VFA concentration during fermentation.

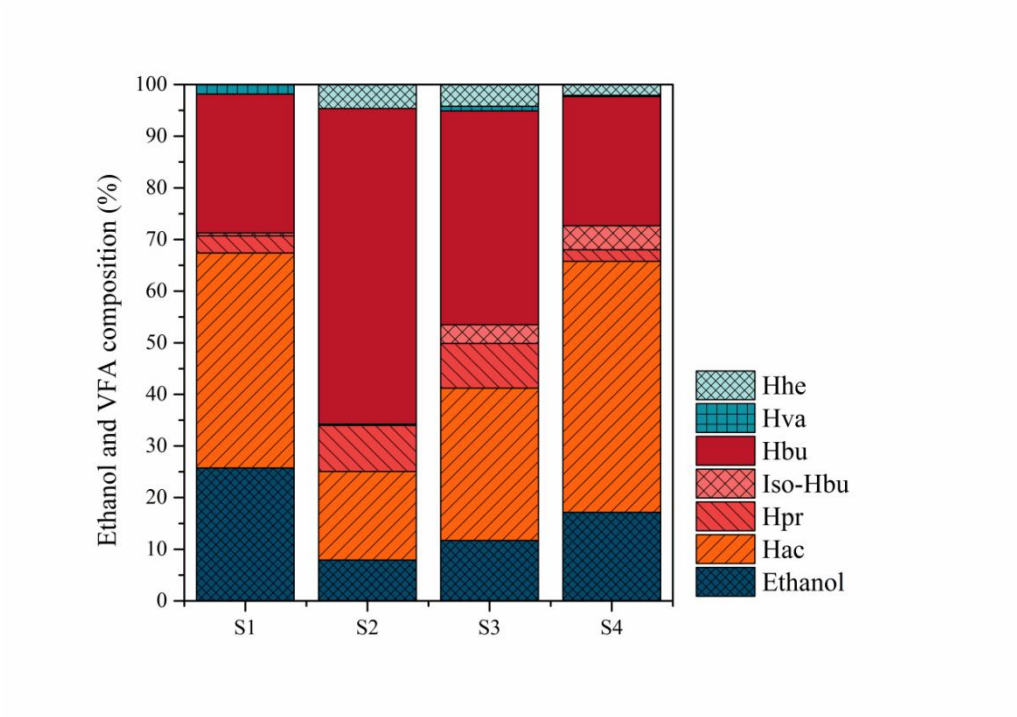

Fig. 5. Distribution of ethanol and individual VFAs in the products.

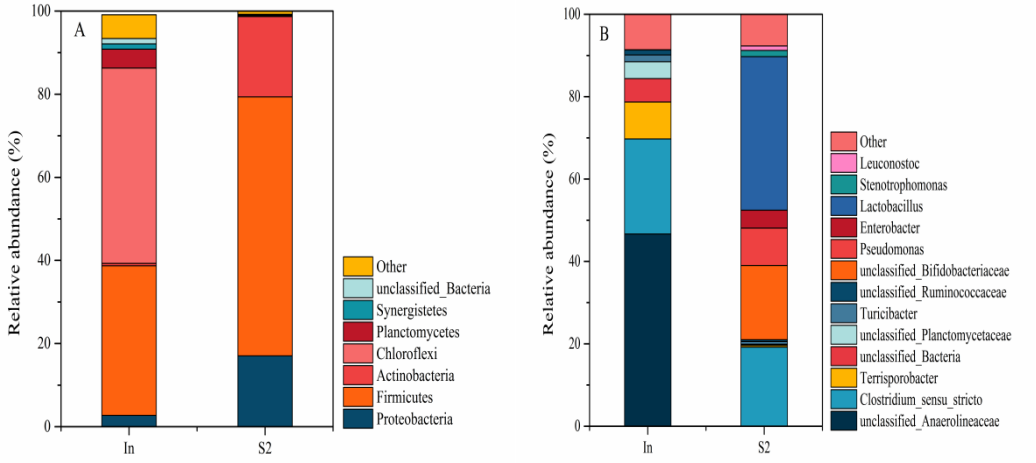

Fig. 6. Relative abundance of dominant species in bacterial community structure (In and S2): at the phylum (A); at the genus (B).

Table 1 Characteristics of the VWs and inoculum.

Parameter	Potato peels	Carrots waste	Chinese cabbage waste	Celery waste	Seed sludge
TS (%)	15.70±1.12	11.78±0.65	4.14±0.35	6.51±1.02	15.21±2.12
VS (%)	14.20±1.82	11.01±0.76	3.36±0.51	5.18±0.35	7.16±0.42
VS/TS (%)	90.45±2.25	93.46±2.58	81.16±3.49	79.57±4.01	47.07±1.69
TCOD (mg/L)	23911±133	19032±201	26593±312	18557±125	71481±156
SCOD (mg/L)	8828±101	9577±95	12794±111	886±121	6948±65

Note: All parameters are based on wet weight.

Table 2 Proportions of the VWs pulp

Fermentation groups	Carrot waste	Potato peel	Celery waste	Chinese cabbage waste
S1 (50% Carrot)	50.0%	16.7%	16.7%	16.7%
S2 (50% Potato peel)	16.7%	50.0%	16.7%	16.7%
S3 (50% Celery)	16.7%	16.7%	50.0%	16.7%
S4 (50% Chinese cabbage)	16.7%	16.7%	16.7%	50.0%

Table 3 Comparison of the diversity of bacterial community in different samples.

Samplegroup	OTUs	Ace	Shannon	Simpson	Coverage
In	313.0	325.60	2.60	0.22	1
S2	242.0	261.83	2.79	0.12	1

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