Nitrogen removal enhancement using lactic acid fermentation products from food waste as external carbon sources: performance and microbial communities

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Abstract: In this study, nitrogen removal using the lactic acid fermentation products from food waste and other external chemical carbon sources (sodium acetate, sodium lactate and starch) was investigated. Similar to sodium acetate and lactate, the lactic acid-enriched fermentation liquid from food waste (FLFW) exhibited a high denitrification rate (5.5 mg NO_x-N/(g-VSS·h)) and potential (0.16 g NO_3^--N/g COD), and could achieve high NH_4^+-N and total nitrogen (TN) removal efficiencies during long-term operation. Using FLFW as supplementary carbon sources reduced the extracellular polymeric substances (EPS) content, improved the settleability and achieved a satisfactory biomass yield of activated sludge. Additionally, the increased microbial metabolic activity and bacterial community diversity and the accumulation of unique bacteria in the activated sludge cultured with FLFW further promoted the organics utilization rate and nitrogen removal efficiency, indicating that the FLFW prepared from solid waste was an ideal

carbon source for wastewater treatment.

Keywords: Nitrogen removal; lactic acid-enriched fermentation liquid from food waste; carbon source; extracellular polymeric substances (EPS); microbial community

1. Introduction

The excessive release of nitrogen into aquatic systems leads to eutrophication problems that impair the survival of aquatic plants and other organisms, therefore, stricter wastewater treatment plant (WWTP) discharge standards have been implemented in China to increase effluent quality, especially with regard to nitrogen content. Among the various nitrogen removal methods, activated sludge (AS) process is extensively applied to wastewater treatment owing to its efficient and reliable performance. However, in biological nitrogen removal (BNR) processes, denitrification is restricted by the insufficient organic matter conditions that significantly limit the nitrogen removal efficiency, resulting in an effluent with high nitrate content (Li et al., 2016a; Zhang et al., 2016a; Sun et al., 2017; Tang et al., 2017a). To improve the total nitrogen (TN) removal efficiency, methods such as optimizing the operational parameters of the treatment systems, exploiting new treatment processes and supplementing external carbon sources are often adopted to enhance denitrification processes (Chu and Wang, 2011; Dai et al., 2015; Frison et al., 2013; Zhu et al., 2009).

Among the aforementioned nitrogen removal enhancement methods, the addition of external carbon source has recently received great attention. A variety of chemicals, such as acetate, alcohol, glucose and starch, have been utilized as efficient carbon sources for promoting nitrogen removal efficiencies. However, it is difficult to accurately control the dosage of the external

carbon source, because an insufficient dosage of carbon source would lead to uncompleted denitrification, while an excessive dosage may increase the chemical oxygen demand (COD) concentration in the effluent (Boley et al., 2000; Chu and Wang, 2011). Excluding the organic matter mentioned above, biodegradable polymers has also been utilized to enhance denitrification, as the amount of organic carbon released by the polymers could be regulated by bacteria responding to the concentration of nitrate in the aqueous phase (Chu and Wang, 2011; Li et al., 2016a). However, the cost of using these chemical carbon sources is often high, which limits their application in practice. To recycle organic solid waste into wastewater treatment and reduce the operation cost, excess activated sludge and agricultural wastes have been directly supplied as carbon sources to promote the denitrification rate (Sun et al., 2017; Yang et al., 2015). Fermentation products from organic solid wastes have also been successfully utilized as supplementary carbon sources in wastewater treatment systems (Li et al., 2016b; Tang et al., 2017a; Zhang et al., 2013; Zhang et al. 2016b). During the fermentation processes, organic wastes are first hydrolyzed into dissolved organic matter and then acidified into organic acids, which allow microorganisms to use solid wastes, thus enhancing denitrification. Our previous studies have identified that organics (organic acids and soluble carbohydrates) in the fermentation liquid from food waste (FLFW) are easily biodegradable carbon sources, and could increase microbial diversity and obviously promote the nitrogen removal efficiency (Tang et al., 2017a; Zhang et al., 2016b). In lab-scale sequence batch reactors (SBR) and pilot-scale membrane bioreactor (MBR) systems, FLFW can be effectively utilized as a carbon source for enhancing nitrogen removal during long-term operation (Tang et al., 2017a; Zhang et al., 2016a).

On the other hand, food waste is a typical organic municipal solid waste (OMSW), causing

significant environmental degradation, and has hindered the development of cities due to its high organics content and biodegradability (Han et al., 2016; Zhou et al., 2017). Traditional methods of food waste disposal, such as landfill, composting and incineration, have become less feasible due to public concerns of surrounding sustainability and the stringency of environmental standards (Zhou et al., 2017). Thus, developing environmentally sustainable and effective solutions of treating the increasing volume of food wastes has received great attention. Anaerobic fermentation is a suitable method of stabilizing solid organic wastes, such as food waste, and it can produce biogas or other intermediates as energy sources (Tang et al., 2016; Zhou et al., 2017). Volatile fatty acids (VFAs), lactic acid and alcohols are important intermediates in anaerobic food waste fermentation, and could be effectively utilized as carbon sources for nutrient removal (Tang et al., 2017a; Zhang et al., 2016b). Thus, using fermentation products as carbon sources to enhance nutrient removal could be a solution to food waste disposal and wastewater treatment issues. According to our latest results, lactic acid production can be achieved at a lower pH (4-5) and within a shorter fermentation period than VFAs obtained from food waste fermentation (Tang et al., 2017b). Thus, using food waste for lactic acid fermentation and applying lactic acid-enriched products as carbon sources for wastewater treatment would be economical for both food waste disposal and wastewater treatment.

Although lactic acid originated from industrial wastewater has been utilized as carbon sources for nitrogen removal by some researchers (Fernándeznava et al., 2010; Sage et al., 2006), to the best knowledge of the authors, the use of lactic acid fermentation products to enhance nitrogen removal has not yet been recorded. Thus, in this study, the effects of lactic acid-enriched FLFW as an external carbon source to promote nitrogen removal were investigated. The properties of the

activated sludge and microbial community structures were also discussed to provide a new combined method of solid waste disposal and wastewater treatment.

2. Materials and methods

2.1. Preparation of the FLFW

Fresh food waste was collected from the canteen of a university in Xi'an, China. The food waste was mainly consisted of rice, vegetables and meat. After removing animal bones, waste tissues and clamshells, the food waste was crushed using an electrical blender. This slurry was sieved (1 mm) and subsequently stored in a refrigerator (4°C) for future use. Before the slurry was added to the fermentation reactor, its total solids (TS) content was adjusted to approximately 8-10% using tap water. And then 20 L of the prepared slurry was added into a continuous stirring tank reactor (CSTR) and fermented anaerobically at 37°C with intermittent pH adjustment (once every 12 h) to 6 as reported in the previous studies (Tang et al., 2016). The fermentation broth was sampled and the COD, carbohydrate, protein, lactic acid, acetate, propionate and butyrate were measured daily following the methods described in the previous studies (Tang et al., 2016). After 5-6 days, the content of the components in the fermentation products stabilized and the lactic acid constituted approximately 75-80% of the soluble chemical oxygen demand (SCOD) (Supplementary Information). The fermented slurry was discharged from the reactor, and centrifuged at 5000 rpm for 10 min, the supernatant was filtered through a 0.45-μm membrane, and then refrigerated (4°C) for future use. The main organic components of the FLFW are shown in Table 1.

Table 1.

2.2 Continuous operation of the SBR with FLFW as carbon source

To explore the effects of external carbon sources on nitrogen removal, the characteristics of activated sludge and microbial communities in the activated sludge, FLFW and three other chemicals (sodium acetate, sodium lactate and starch) as substitutes of the main organic components of FLFW were respectively supplied as the sole organic carbon sources in four SBRs (working volume of 5 L). The raw activated sludge used in the reactors was obtained from the aerobic and anaerobic tanks of a full-scale anaerobic/anoxic/oxic-membrane bioreactor system that treats municipal wastewater with a TN removal rate exceeding 80%. The influent of the SBRs was synthetic wastewater with tap water supplied alongside the four carbon sources and other trace elements, as reported by Zhang et al. (2016a). The operation sequences of the SBRs were the same as those presented in our previous studies and are provided in the Supplementary Information. The COD of the influent remained at 300 mg/L, while the NH_4^+-N content increased from 20 to 40 mg/L during operation period. The hydrolytic retention time (HRT) and sludge retention time (SRT) of all reactors were 10 hours and 30 days, respectively. The mixed liquid suspended solids (MLSS) in all reactors remained at 3000-5000 mg/L, and the ratio of mixed liquid volatile suspended solids (MLVSS) to MLSS was approximately 0.7-0.8.

2.3 Nitrogen uptake rate (NUR) tests

To elucidate the denitrification properties of the FLFW, a series of NUR tests were conducted following the methods presented in the previous studies (Tang et al., 2017a; Zhang et al., 2016b; Sage et al, 2006). First, activated sludge was sampled from the SBRs into four flasks, and washed with tap water three times to remove residual nitrogen compounds and organic matter from the sludge. The activated sludge in each flask was then diluted with tap water to make up a volume of 1 L and obtain a MLVSS of approximately 3 g/L. Oxygen in the mixed liquor was flushed with

nitrogen gas (N_2). Thereafter, sodium nitrate was added into each flask to generate a final NO_3^--N content of 50 mg/L, FLFW and other three chemicals (sodium acetate, sodium lactate and starch) were then added to obtain a COD content of approximately 300 mg/L. A control test for each sludge was also conducted with 50 mg/L of NO_3^--N but no additional carbon source was added. The flasks were then sealed and the liquor was mixed with stirrers. 20 mL mixed liquor was periodically sampled from each flask to analyze the NO_3^--N, NO_2^--N and COD contents. The specific denitrification rate (SDNR), denitrification potential (DP), anoxic sludge yield and COD utilization efficiency for nitrogen removal were calculated according to Sage et al. (2006).

2.4. Analysis methods

2.4.1. Extraction and measurement of EPS

To explore the effect of carbon sources on the release of EPS. EPS (both soluble EPS (SEPS) and bound EPS (BEPS)) were extracted from activated sludge using a thermal treatment method according to Hu et al. (2013). The contents of EPS samples were analyzed in terms of proteins and polysaccharides. Polysaccharides were detected using the anthrone method, in which glucose was used as a standard (Frølund et al., 1996), and proteins in the EPS were quantified using a modified Lowry method, with bovine serum albumin (BSA) as the standard reference (Lowry et al., 1951).

2.4.2. Gel filtration chromatography (GFC) analysis

The molecular weight distributions (MWD) of the EPS samples were analyzed using a GFC analyzer (LC-2010A, Shimadzu Corporation, Japan) equipped with a Zenix SEC-100 type gel column (Sepax Technologies Corporation, USA) and a UV detector (SPD-10, Shimadzu Corporation, Japan) at 40°C. 150 mM sodium phosphate buffer (Na_2HPO_4:NaH_2PO_4=1:1) was adopted as the eluent at a flow rate of 1.0 mL/min. The injection volume was 10 μL for SEPS, and

lower (2 μL) for BEPS.

2.4.3. Three-dimensional excitation-emission matrix fluorescence spectra (3D-EEM)

To investigate the dissolved organic matter (DOM) in the supernatant, samples collected from reactors were centrifuged (5000 rpm for 10 min, 4°C) and filtered through 0.22-μm filters. The filtrate was subsequently measured using a FP-6500 spectroflurometer (Jasco Corporation, Japan). The emission spectra of the EEM were scanned from 220 to 550 nm in 5 nm increments by varying the excitation wavelength from 220 to 550 nm in 5 nm steps.

2.4.4. Particle size distribution (PSD) analysis

PSD of the sludge samples from the four SBRs was measured using a laser granularity distribution analyzer (LS 230/SVM+, Beckman Coulter Corporation, USA), which had a detection range of 0.4-2000 μm and showed a good accuracy and reproducibility. In this study the typical PSD curves were reported.

2.4.5. Biolog plate analysis

The metabolic characteristics of microorganisms in activated sludge acclimated with different carbon sources were assessed using the Biolog-ECO plates (Biolog, Inc., Hayward, CA, USA) as it was described by Kong et al. (2013) and in our previous studies (Tang et al., 2017a). Briefly, the activated sludge samples from the four reactors were diluted to 1:1000 with sterilized NaCl solution (0.9 %, w/v) and shaken four times for 15 s each. The resulting suspension of 1 mL was diluted with the saline solution to control the optical density (OD) close to 0.05 at 600 nm, which ensured that the four sample solutions contained approximately the same biomass concentration. Then 150 μL of the diluted mixture was added to the Biolog plate well using eight channel pipettes and the plates were inoculated at 25°C in darkness. The absorbance (OD 590 nm) of the

wells was recorded for 216 h using an ELISA plate reader at every 24-h interval. The data analysis processes were according to the previous study (Zhang et al., 2016a).

2.4.6. Microbial community analysis

Microbial communities in the sludge cultured with the four carbon sources were further analyzed using the high throughput sequencing technology. Activated sludge samples were sent to Sangong, Inc. (Shanghai, China) for DNA extraction and the next-generation sequencing processes. The extracted DNA was amplified by polymerase chain reaction (PCR) using the primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') for the V1-V3 region of the 16S rRNA genes (Luo et al., 2017; Tang et al., 2017a). Pyrosequencing was then conducted using an Illumina MiSeq platform. The homologous or ambiguous sequences or those with a length less than 300 bp were trimmed to obtain high-quality sequences with an average length larger than 500 bp for the taxonomic classification.

2.5 Additional chemical analysis methods

Influent, effluent and mixed liquor were periodically sampled from reactors. The nitrogen compounds (NH_4^+-N, NO_3^--N, NO_2^--N and TN), COD, PO_4^{3-}-P, MLSS, MLVSS, biomass yield and sludge volume index (SVI) were measured according to standard methods (APHA, 2012). The capillary suction time (CST) was detected with a CST meter (DPDFC-10A, China).

3. Results and discussion

3.1 Overall nitrogen removal performance

3.1.1 Denitrification properties of the FLFW

To investigate the nitrogen removal properties of the fermentation products and compare the

denitrification rate and potential with other chemical carbon sources, a series of NUR testes were conducted. Variations in the NO_3^--N and NO_2^--N concentrations are shown in Fig. 1. It was clearly found that the content of NO_3^--N in the reactors added with sodium acetate and lactate decreased rapidly, and reached a constant value within 90 and 105 min, respectively, indicating a relatively high denitrification rate. Similar to acetate and lactate, a rapid decrease in the NO_3^--N content and a residual concentration of 0.5 mg/L were also observed in the reactor using FLFW as carbon source (Table 2). Lactate and VFAs in the FLFW could be effectively utilized as carbon sources by denitrifiers, which is consistent with the findings of the previous studies (Tang et al., 2017a; Zhang et al., 2016b). However, starch is a macromolecular organic matter and is utilized by bacteria through complicated metabolic processes. Thus, a slow reduction of NO_3^--N was observed in the reactor supplied with starch, and the final NO_3^--N concentration was 32.3 mg/L, indicating a weak denitrification, which further demonstrated the advantages of the carbon source produced by anaerobic fermentation, because starch is the primary component of food waste. Additionally, due to the lack of available organics for denitrification in the control flasks, NO_3^--N content only decreased slightly (Supplementary Information), indicating that the reductions of NO_3^--N could be attributed to the addition of external carbon sources to the flasks.

In the reactor supplied with acetate, NO_2^--N sharply increased and achieved the maximum value of 17.8 mg/L within 45 min, which could be explained by the competition for electrons between the nitrite reductase and nitrate reductase (Ge et al., 2012). Acetate is a readily biodegradable carbon source that could rapidly provide energy for bacteria and exhibit a high denitrification rate and NO_2^--N accumulation (Zhang et al., 2016b). After rapid accumulation, the NO_2^--N content gradually decreased to 0.05 mg/L within approximately 120 min. However, NO_2^--N content in

other reactors was very low, indicating slight NO_2^--N accumulation during denitrification.

Fig. 1.

The parameters of the denitrification processes could be obtained from the variations in nitrogen compounds and organic matter contents (Table 2). It was found that, similar to sodium acetate and lactate, FLFW exhibited a high denitrification rate (5.5 mg NO_x-N/g-VSS·h), which was much higher than that of starch (0.8 mg NO_x-N/g-VSS·h). Additionally, FLFW and lactate showed the same denitrification potential (DP) (0.16 g NO_3^--N/g COD), and was a little lower than that of acetate (0.20 g NO_3^--N/g COD). This could be because the lactic acid is the main component of FLFW, thus, both exhibited similar denitrification capacities. Starch had the lowest DP (0.07 g NO_3^--N/g COD) and COD utilization efficiency (20.7%), which was because starch is a high-molecular organic matter and COD loss in the form of microbial competition (fermentation/denitrification) and electron partition through assimilation (biomass production) and dissimilation (energy production by denitrification) is greater when it is metabolized (Sage et al., 2006). The highest anoxic sludge yield (0.79 g/g-COD) was observed in the reactor fed with starch, further demonstrating that COD loss was higher and denitrification potential was lower in this reactor. Due to the high DP, the sludge yields in reactors with acetate and FLFW was relatively lower, further indicating their high organics utilization efficiencies for nitrogen removal (Guo et al., 2017).

Table 2.

3.1.2 Nitrogen removal during long-term operation

To further explore the nitrogen removal performance during long-term operation, four SBRs were operated using the four carbon sources, respectively (Fig. 2). Regardless of the NH_4^+-N

content in the influent, NH_4^+-N concentrations in the effluent of each reactor during the operation period were very stable and below 0.5 mg/L. However, NH_4^+-N in the effluent of reactors with lactate and starch was unstable during the first 10 days, which could be because the microorganisms, especially the nitrifying bacteria, were influenced by the addition of these two carbon sources. However, after a short time, NH_4^+-N concentration gradually decreased and stabilized. Obviously, acetate and FLFW could be easily accommodated by the microorganisms and had little negative influence on the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) as the NH_4^+-N content in effluent was stable from the beginning of the operation.

However, the NO_3^--N and TN content in effluent showed some distinctions (Fig. 2b and c). Lower NO_3^--N content (< 5 mg/L) was detected in the effluent of reactor with lactate and acetate showing a TN removal efficiencies of 80-90%. Reactor fed with starch exhibited a very unstable removal efficiency during the early stages, which further demonstrated its negative effect on nitrification and denitrification, owing to its high molecular weight, and that bacteria would require a longer time to adjust the metabolism. Although fluctuations occurred due to the increase in nitrogen loading (from 30 to 40 mg/L) and a lower COD to nitrogen ratio (7-8) from Day 50 to 70, the reactor supplied with FLFW also achieved stable nitrogen removal. The NO_3^--N content in effluent gradually decreased and maintained below 2.5 mg/L, showing a TN removal efficiency above 85%. Due to the low organic nitrogen content in the carbon sources, TN content of the influent was very close to that of NH_4^+-N. Similar to acetate and lactate, the TN content in effluent from the reactor with FLFW was lower than that in effluent from reactor supplied with starch, indicting a higher TN removal efficiency.

During the long operation period, similar to other easily biodegradable organics (acetate and

lactate), organics in FLFW could be effectively utilized by bacteria in denitrification process. Furthermore, the FLFW had a slightly negative influence on nitrification as it has been discussed in the previous studies (Tang et al., 2017a). Overall, the lactic acid-enriched FLFW could significantly enhance the denitrification and promote nitrogen removal during wastewater treatment.

Fig. 2.

3.1.3 Characteristics of EPS with FLFW as carbon source

EPS (both SEPS and BEPS) are influenced by the carbon sources in the influent and have an important effect on the characteristics of activated sludge, such as settleability, dewaterability and sludge flocks structure and stability (Reid et al., 2008; He et al., 2018). Thus, EPS in the activated sludge were analyzed. The SEPS in reactor supplied with FLFW was higher than that in the reactors supplied with acetate and starch (Table 3), while BEPS (32.9 mg/g-VSS) was the lowest among the four carbon sources. This could be because protein was the component of FLFW, which was not fully utilized by bacteria and accumulated in the supernatant (Tang et al., 2017a; Zhang et al., 2016a). However, SEPS in reactor with lactate was the highest (16.2 mg/g-VSS), which could due to the bacterial protection from extreme environmental conditions or a shift in community structure (He et al., 2018). Additionally, the BEPS in sludge with lactate was very high, further verifying the increased SEPS content in the supernatant, as BEPS would release from the bacterial cells to supernatant (Meng et al., 2009). Although the SEPS in reactor with acetate was the lowest, BEPS was relatively high (51.3 mg/g-VSS). Protein in the SEPS could have originated from the cell lysis and entrapped exoenzymes (Liu and Fang, 2003), and was the main component of all samples. The preferential production of protein over polysaccharide could contribute to the

maintenance of the matrix structure and stability (He et al., 2018), which is important for stable reactor operation. Additionally, the molecular weight distribution profiles of the SEPS and BEPS were significantly different between the four reactors (Supplementary Information), indicating that the EPS components were also affected by the carbon sources.

Table 3.

The fluorescent characteristics of the dissolved organic matter (DOM) were further analyzed using the 3D-EEM (Fig. 3). According to the previous studies (Chen et al., 2003), peak A (Ex/Em=230 nm/310-380 nm) represents aromatic protein-like substances, peak B (Ex/Em=275-290 nm/335-370 nm) reflects tryptophan protein-like substances, and peak C (Ex/Em=310-350 nm/405-440 nm) indicates the existence of humic acid-like substances.

The chemical composition of soluble microbial products (SMP) is highly variable and dependent upon, amongst other factors, microbial diversity and physiology (Reid et al., 2008). In the reactors using lactate and acetate as carbon sources, both peak A and B were detected, indicating the presence of both aromatic and tryptophan protein-like substances in the samples. However, in the reactor added with starch, only peak B was present in the supernatant, indicating a weak production of aromatic protein-like substances, which could be due to differences in the metabolic pathways between starch and the other three carbon sources. FLFW is a complex carbon source containing low-molecular (lactate and acetate) and macromolecular (carbohydrate and protein) organics, thus, the degradation processes would be more complicated and produce different metabolites (Maqbool et al., 2017). Peaks A and B were detected at a high intensity in the samples from reactor with FLFW, showing a higher content of protein-like substances. Additionally, it was noticed that the positions of peaks A and B changed, indicating differences in

the structures of the organics in the supernatant, which could be further verified by the MWD profiles in the Supplementary Information.

The different properties of fluorescence between DOM implied that the carbon sources indeed affected the bacterial metabolites, which could be attributed to the differences in the bacterial communities. This will be discussed in more detail in section 3.2.

Fig. 3

3.1.4 Settleability, dewaterability and biomass yield of activated sludge

It has been reported that dewaterability, in terms of CST, is positively influenced by carbohydrate in SEPS and settleability reflected by SVI is highly correlated with protein in BEPS (Reid et al., 2008). Sludge cultured with lactate exhibited a higher SVI (98.2 mL/g-VSS) and CST (3.44 s.L/g-VSS) than the other sludge (Table 4), which can be explained by the EPS components in Table 3. Although lactic acid was the main component of the FLFW, the activated sludge cultured with FLFW exhibited a lower SVI (88.7 mL/g-VSS) than that fed with lactate, which could be due to the existence of other organics (acetate, propionate, carbohydrate) in the FLFW or different microbial communities in the activated sludge. In addition, the particle size distributions (PSD) of the four sludge were different (Supplementary Information). Mean size of the sludge flocs cultured with starch was the largest, which further explained the lowest SVI value (Table 4). The PSD profiles of the activated sludge cultured with FLFW were very close to those with sodium lactate and acetate, further demonstrating that they had similar mean particle sizes.

Biomass yield is an important parameter for describing the properties of carbon sources. In general, a higher biomass yield indicates that more carbon sources are utilized to produce biomass and less are used in the denitrification processes (Guo et al., 2017). Moreover, the disposal of

excess sludge requires money and energy (Amanatidou et al., 2015). Thus, biomass yield is an important indicator for assessing the availability and effectiveness of external carbon sources (Fernándeznava et al., 2010). Among the four carbon sources, starch yielded the most biomass (0.48 g-VSS/g-COD). While the biomass yield of reactor with FLFW was approximately 0.41 g-VSS/g-COD, which is slightly higher than that of the reactor added with acetate and lactate, but this value is still within the range reported by other researchers (0.3-0.6 g-VSS/g-COD) in wastewater treatment. Thus, using the FLFW as an external carbon source for enhancing nitrogen removal could not only achieve a high nitrogen removal efficiency, but also low excess sludge production.

Table 4.

3.2 Microbial community analysis

According to the earlier discussions, the reactors with different carbon sources exhibited differences in pollutants removal and sludge properties. It has been reported that carbon sources not only influence microbial metabolism, but also the bacterial community structures, which also affects the pollutants removal. To further reveal the effect of carbon sources on microbial communities, the characteristics of bacterial metabolism and microbial diversity in the four reactors were investigated.

3.2.1. Microbial metabolic properties

To characterize the metabolic activity of microorganisms in activated sludge, variations in the average well-color development (AWCD) during incubation were investigated using the Biolog ECO-plate. As shown in Fig. 4a, the AWCD value increased slightly during the first 24 h, which could be because the microorganisms needed time to accommodate new environment and the

ECO-plate. After a short-time of modulation, the AWCD value rapidly increased and approached the constant values after 192 h in all samples.

The final AWCD value was significantly different between the four samples. The AWCD value of the sludge fed with lactate increased slowly and reached the lowest value of 0.66. While the sludge sample from reactor with FLFW achieved the highest AWCD value (0.95), which was higher than that from reactors with starch and acetate, indicating more rapid bacterial growth and higher microbial metabolic activity (Kong et al., 2013). These could be caused by the higher bacterial diversity of the activated sludge (Zhang et al., 2016a). In the FLFW, hydrolysates (such as soluble carbohydrate and protein) and acidogenesis products (such as VFAs and lactate) provided bacteria with more types of carbon sources, thus, a higher microbial diversity was present and involved in substrate degradation (Tang et al., 2017a).

Additionally, the substrates in the ECO-plate can be categorized into alcohols, amines, amino acids, carbohydrates, carboxylic acids, esters and polymers. Qualitative measures of substrates utilization by each group can be calculated from the average absorbency fraction at 192 h in the same manner as those for the whole plate. Although microorganisms in the four sludge samples could utilize all the 7 types of carbon sources, some differences could also be found (Fig. 4b). Bacteria in the sludge from reactor with lactate preferred to utilize amino acids (17.1%) and esters (17.9%), and exhibited low polymers digestion ability (10.4%). While the sludge cultured by starch showed a higher capacity to digest the polymers with a proportion of 14.9%, which was probably due to the fact that starch is a macromolecular organic matter and the bacteria in sludge were accommodated to utilizing polymeric substances. The sludge from the reactor supplied with acetate showed similar metabolic capacity for all the 7 carbon sources.

Microorganisms fed with FLFW exhibited higher capacity in using the amines (17.0%), amino acids (16.9%) and polymers (15.0%), which could be attributed to the existence of protein and carbohydrates in the FLFW that would affect bacterial metabolism or promote the growth of some unique bacteria in the activated sludge (Tang et al., 2017a). Due to the higher proportions of these three carbon sources, other carbon sources (carboxylic acids (10.7%) and alcohols (12.4%)) showed smaller proportion than that in other activated sludge, but this did not mean the lower usage capacity of these carbon sources due to the higher AWCD value than other samples.

Fig. 4c was obtained to further compare the four sludge samples using the McIntosh index (U) to evaluate the diversity of metabolism in multi-dimensional space and the Shannon's diversity (H) to assess species richness (Zhang et al., 2016a). The sludge cultured with FLFW showed the highest U value of 5.7, indicating that the bacteria in the sludge could digest more types and more complicated carbon sources, further verifying the higher AWCD value in Fig. 4a. It was deduced that organics in the FLFW could be utilized as carbon sources by bacteria, or more species of bacteria accumulated in the sludge to utilize a broader range of organics. The sludge cultured with lactate exhibited the lowest diversity (4.1), which might be due to the fact that lactate affected the selection of microorganisms accumulating in the reactor. The activated sludge cultured with acetate and starch exhibited a higher H value than those added with lactate. The higher metabolic diversity of the activated sludge with FLFW could be related to the distinct microbial community, which will be discussed in section 3.2.2.

The Shannon's diversity is related to the types of utilizable carbon sources (Yang et al., 2011; Zhang et al., 2016a). No evident differences were identified in Fig. 4c, indicating that the carbon sources in the plate were almost equally utilizable for the four sludge samples.

Fig. 4.

3.2.2. Microbial community analysis

Microbial communities in the activated sludge were investigated using the high-throughput sequencing technology. The rarefaction curves of the four samples drawn from each of the units reached a plateau (Supplementary Information), indicating that all bacterial communities were characterized in this study. The number of operational taxonomic units (OTUs), Good's coverage, the Shannon diversity, Chao1, and ACE indices, as well as the Simpson index at a cutoff level of 3% were calculated (Table 5). The Good's coverage of the four samples was 99%, demonstrating that the sequence libraries constructed in this study covered the diversity of the whole microbial community. The number of OTUs in the samples ranged from 953 to 1233. The greater microbial richness in the sludge acclimated with FLFW than that of the other activated sludge was evidenced by the Chao 1 estimator of the total OTU numbers and validated by the respective ACE indices, further verifying the higher metabolic activity in Fig. 4. Similar to the Simpson indices, the Shannon indices ranged from 3.5 to 4.4, demonstrating that the microbial diversity of the four samples did exhibit some differences (Table 5).

Table 5.

The Bray tree of the microbial communities in the four samples was obtained based on the OTUs (Fig. 5a). Although lactic acid was dominant in the FLFW, the microbial communities in reactor with FLFW were more similar to those in reactor with acetate, which could be because other organics (such as carbohydrate, protein and VFAs) also coexisted in the FLFW, and a variety of bacteria would accumulate in the sludge. Obviously, the OTUs in the sludge cultured with starch were very different to those in the other three samples, implying greater differences in its

microbial communities.

The VEEN diagram further described the relationships between the four samples (Fig. 5b). Only 104 OTUs were shared by all the four samples, accounting for 8.4%-10.9% of the OTUs in each sample. This indicates little similarity between the microbial communities acclimated by the four carbon sources. OTUs in the sludge cultured with FLFW and acetate were 482, accounting for 39.1% of the OTUs in the sludge added with FLFW, which indicated that high similarity in microbial communities existed in the two sludge. In addition, the sludge acclimated with FLFW and lactate shared 285 OTUs (approximately 29.9% of the OTUs in the sludge with lactate), which further explained their close distance in the Bray tree (Fig. 5a).

Fig. 5.

Fig. 6.

By further classifying the OTUs, it was found that Proteobacteria, Bacteroidetes and Firmicutes were the main phyla of all samples (Fig. 6a). In the sludge supplemented with starch, the relative abundance of Proteobacteria (19.7%) and Firmicutes (0.7%) were very low, but that of Bacteroidetes was very high, with a proportion of 49.5%. It was reported that Bacteroidetes could intervene in the degradation of proteins and can ferment amino acids to acetate (Riviere et al., 2009). Candidate-division-TM7, with a relative abundance of 15.1%, was the almost unique phylum in the sludge added with starch. Chloroflexi, with a proportion of 3.8%, were much more abundant in the sludge with starch than they were in other samples. Chloroflexi is responsible for the degradation of soluble microbial products (SMP) and other complicated substrates (Miura et al., 2007), thus, the higher abundance of this phylum in the sludge further testified the low EPS content in the sludge and the effective degradation of starch.

Microorganisms selectively accumulated in the sludge with sodium lactate as carbon source. Proteobacteria, Bacteroidetes and Firmicutes were the dominant phyla with a relative abundance of 70.2%, 19.8% and 8.8%, respectively, and these three phyla accounted for a proportion of 98.8%. While sludge fed with acetate and FLFW exhibited similar microbial community structures. Proteobacteria (47.2% and 46.7%), Bacteroidetes (19.4% and 30.5%) and Firmicutes (26.7% and 12.6%) were detected in both sludge, which further verified their close relationship in the Bray tree. Additionally, the relative abundance of Chloroflexi in both sludge was very similar, but Verrucomicrobia (3.8%) was observed at a greater abundance in the sludge with FLFW. Other phyla, such as Planctomycetes, Actinobacteria and Acidobacteria were also detected, though they were present at lower proportion. The relevant abundance of Nitrospirae was higher in sludge added with FLFW (0.4%) and acetate (0.5%), further explaining the stable nitrification rate during the operation (Tang et al., 2017a).

At the family level (Fig. 6b), Hydrogenophilaceae, a family of Betaproteobacteria, was more abundant in the activated sludge cultured with lactate, with a relative abundance of 36.6%, but it was less abundant in the other sludge (<1%). Rhodocyclaceae and Comamonadaceae were also detected, with a relative abundance of 10.7% and 5.4%, respectively. Of the Bacteroidetes, Flavobacteriaceae (13.9%) was the most dominant member. Christensenellaceae was the most abundant of the Firmicutes. Thus, highly selective enrichment of bacteria was observed in the activated sludge with lactate as a carbon source, which was consistent with the lower OTU number and Shannon diversity index (Table 5).

The main families in sludge acclimated with starch were Flavobacteriaceae (14.6%) and Chitinophagaceae (28.1%), both of which are Bacteroidetes. Additionally, Rhodospirillaceae

(3.8%) and Rhodocyclaceae (3.7%) were the detectable components of Proteobacteria, Roseiflexaceae (3.4%) was the detected member of the Chloroflexi. The diverse microbial structure of the sludge might be attributed to the metabolic properties of starch.

Microbial communities in the sludge sampled from reactor with acetate and FLFW are similar. Rhodobacteraceae was the dominant member of the Alphaproteobacteria in both sludge, accounting for 17.8% and 12.2%, respectively. Of the class Betaproteobacteria, Nitrosomonadaceae (2.5%), Rhodocyclaceae (6.8%) and Comamonadaceae (9.0%) were the main members in sludge acclimated with FLFW. However, in the sludge with acetate, Rhodocyclaceae (13.0%) was the dominant family. Both Rhodocyclaceae and Comamonadaceae could use refractory organics as carbon sources for denitrification (Chung et al., 2009; Sun et al., 2017), further explaining the high metabolic capacity and nitrogen removal efficiencies of these sludge. Similar to the sludge cultured with lactate, Flavobacteriaceae (with a relative abundance of 13.0%) also accumulated in the sludge with FLFW. Saprospiraceae (12.1%) was particularly dominant in the activated sludge supplied with FLFW. Both of these families can hydrolyze and utilize complex carbon sources, which further proved the strong metabolic capacity of sludge with FLFW (McIlroy and Nielsen, 2014; Tang et al., 2017a). Interestingly, Christensenellaceae, a family of Firmicutes that degrades volatile fatty acids (Ferrero, et al., 2012), was less abundant in the sludge with FLFW (12.5%) than in that with acetate (26.6%), but more abundant than in that with lactate (8.7%), which could be because acetic acid existed in the FLFW. Moreover, Verrucomicrobiaceae, a member of Verrucomicrobia, was obviously detected in the sludge with FLFW.

The first 50 genera of the four samples were compared (Fig. 6c). *Flavobacterium* was the dominant genera of all samples. But *Candidatus_Saccharimonas*, with a relative abundance of

15.1%, was uniquely found in the reactor with starch. *Rhodobacter* was mainly observed in the sludge cultured with FLFW, *Thauera* was detected in both reactor with lactate and acetate with appreciable abundance and *Azoarcus* was more abundant in sludge added with acetate. All of these bacteria are dominant in wastewater treatment plants (WWTPs) and are involved in denitrification (Ma et al., 2015). Except for the similar genera in the samples, some bacterial such as *Prosthecobacter*, *Ferrovibrio*, *Candidatus_Accumulibacter* and *Variovorax* were obviously found with higher abundance in the sludge with FLFW than other carbon sources, and they can remove refractory organics and nitrogen (Willems et al., 2015; Gonzalez-Martinez et al., 2016). Additionally, *Nitrospira*, a genus of nitrifying bacteria, showed higher proportion in the sludge cultured with acetate and FLFW than other carbon sources, which further testified the high and stable ammonia removal efficiencies of the reactors.

The distinct communities between the sludge further explained the different effects of carbon source on microbial diversity and pollutants removal efficiencies. Compared with other traditional chemical carbon sources, the higher bacterial diversity and existence of some unique bacteria in sludge with FLFW improved pollutants removal and demonstrated the feasibility of using the FLFW as a carbon source for enhancing nitrogen removal.

4. Conclusions

Similar to acetate and lactate, lactic acid-enriched FLFW as an alternative carbon source could effectively enhance denitrification and enable high nitrogen removal efficiencies during long-term wastewater treatment. Low EPS content and biomass yield, good sludge settleability and dewaterability were exhibited by the activated sludge supplemented with FLFW. Higher microbial diversity and metabolic capacity were found in sludge cultured with FLFW than other chemical

carbon sources. Thus, lactic acid fermentation could be a suitable method of food waste disposal and wastewater treatment and a promising process for large-scale systems, although more effort is still needed for economic assessment and practical implementation.

E-supplementary data for this work can be found in e-version of this paper online.

Acknowledgments

This work was financially supported by grants from the National Program of Water Pollution Control in China (Grant no. 2013ZX07310-001), the Natural Science Foundation of China (No. 51508450), the Program for Innovative Research Team in Shanxi Province (Grant No. 2013KCT-13) and the Fund for Postdoctoral Scientific Research Project of China (Grant no. 2015M582760XB).

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Table 1. Characteristics of the lactic acid-enriched FLFW

Parameters	unit	Value
pH	-	4.6±0.3
COD	g/L	57.6±4.3
Carbohydrate	g-COD/L	4.3±1.0
Protein	g-COD/L	1.1±0.2
Lactic acid	g-COD/L	45.2±2.5
Acetate	g-COD/L	3.0±0.6
Propionate	g-COD/L	0.4±0.1
Butyrate	g-COD/L	1.1±0.2
Others	g-COD/L	2.4±1.0

Table 2. Denitrification properties of the four carbon sources in the NUR tests

	COD_0	COD_e	NO_3-N_0	NO_3-N_e	SDNR	DP	Sludge yield	COD utilization rate
	mg/L				mg NO_x-N/g-VSS·h	g NO_3-N/g COD	g COD/g COD	%
Starch	300	21.1	52.5	32.3	0.8	0.07	0.79	20.7
Lactate	325.0	13.5	48.9	0.4	5.1	0.16	0.55	44.5
Acetate	267.9	18.1	49.4	0.4	6.9	0.20	0.44	56.1
FLFW	332.6	13.5	51.7	0.5	5.5	0.16	0.54	45.9

Note: NO_X-N means NO_3^--N+0.6NO_2^--N (Sage et al., 2006), COD_0 and NO_3^--N_0 represent the initial content of COD and NO_3^--N, COD_e and NO_3^--N_e indicate the content of COD and NO_3^--N at the end of the tests.

Table 3. SEPS and BEPS concentrations in the activated sludge with the four carbon sources

Unit: mg/g-VSS

	SEPS			BEPS		
	Polysaccharide	Protein	Total	Polysaccharide	Protein	Total
Starch	0.5±0.1	7.1±2.9	7.6±2.8	14.9±5.2	23.7±2.2	37.3±7.1
Lactate	1.4±0.2	14.8±1.9	16.2±1.9	16.2±4.2	33.2±1.0	51.9±7.7
Acetate	0.5±0.2	6.4±2.4	6.8±2.3	22.7±8.7	25.9±5.2	51.3±4.9
FLFW	0.4±0.2	10.4±3.2	11.2±3.5	11.6±1.9	21.3±0.4	32.9±2.1

Table 4. Characteristics of the activated sludge cultured with the four carbon sources.

	SVI (mL/g-VSS)	CST (s.L/g-VSS)	Biomass yield (g VSS/g-COD)	Particle size (μm)
Starch	65.5±16.8	2.51±1.05	0.48±0.06	99.5
Lactate	98.2±22.5	3.44±1.24	0.34±0.10	65.5
Acetate	70.4±8.6	2.53±1.29	0.39±0.03	69.9
FLFW	88.7±15.3	2.84±0.56	0.41±0.07	69.7

Table 5. Microbial diversity index of the sludge with different carbon sources.

Sample	Sequences	OTUs	Shannon index	ACE index	Chao1 index	Coverage	Simpson
Starch	31939	1011	4.2	1225.5	1141.0	0.99	0.06
Lactate	24499	953	3.5	1158.9	1050.9	0.99	0.14
Acetate	24282	1223	4.1	1487.8	1373.8	0.99	0.09
FLFW	26428	1233	4.4	1583.6	1439.0	0.99	0.05

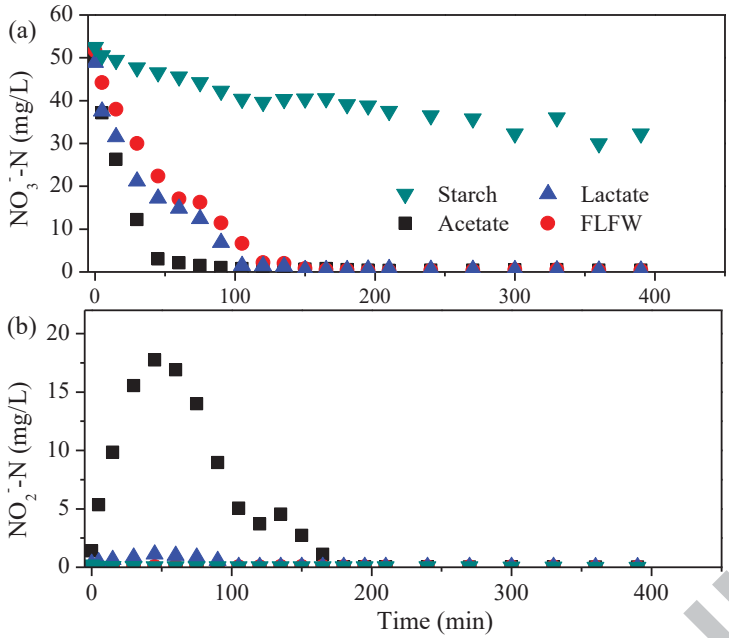

Fig. 1. Variations of NO_3^--N and NO_2^--N in the NUR tests with the four carbon sources

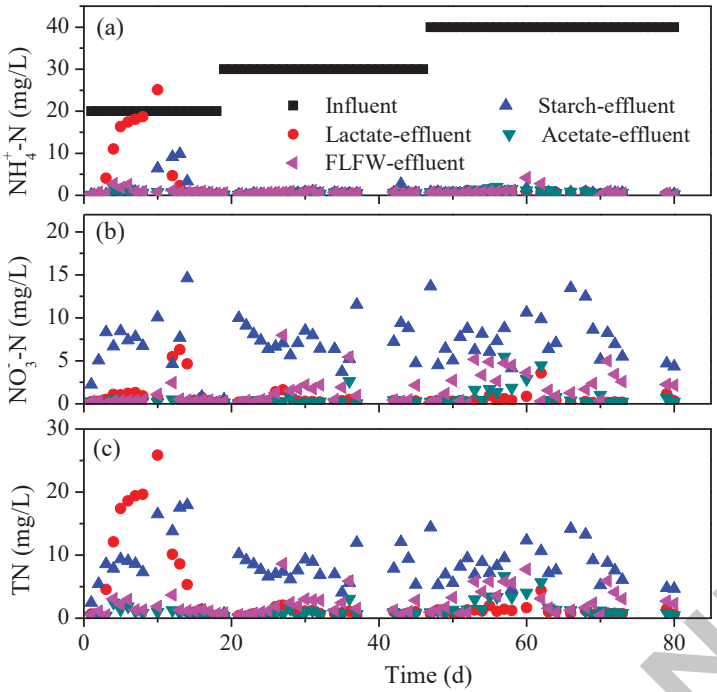

Fig. 2. NH_4^+-N, NO_3^--N and TN content in influent and effluent of the four reactors during the long-term operation

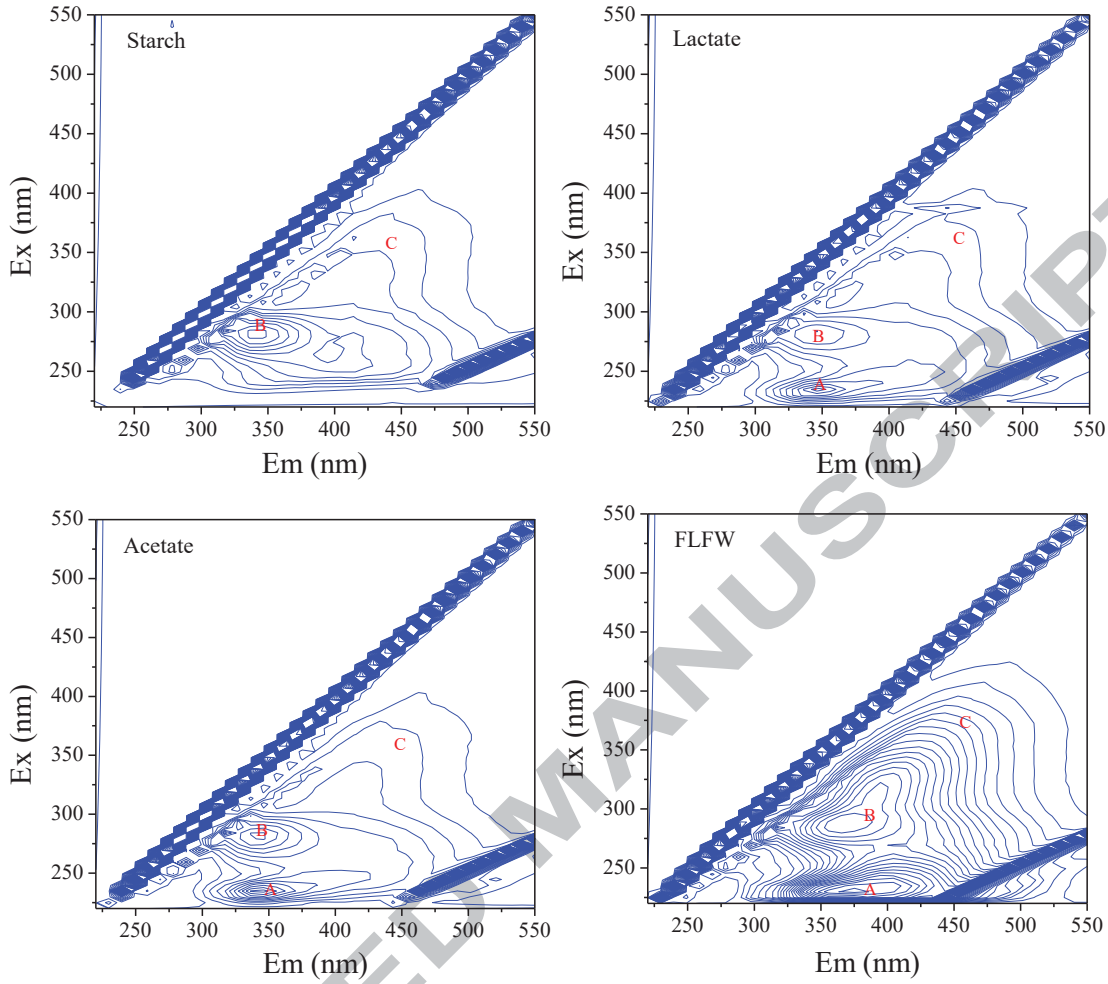

Fig. 3. EEM fluorescence spectra of the DOM in the supernatant with the four carbon sources

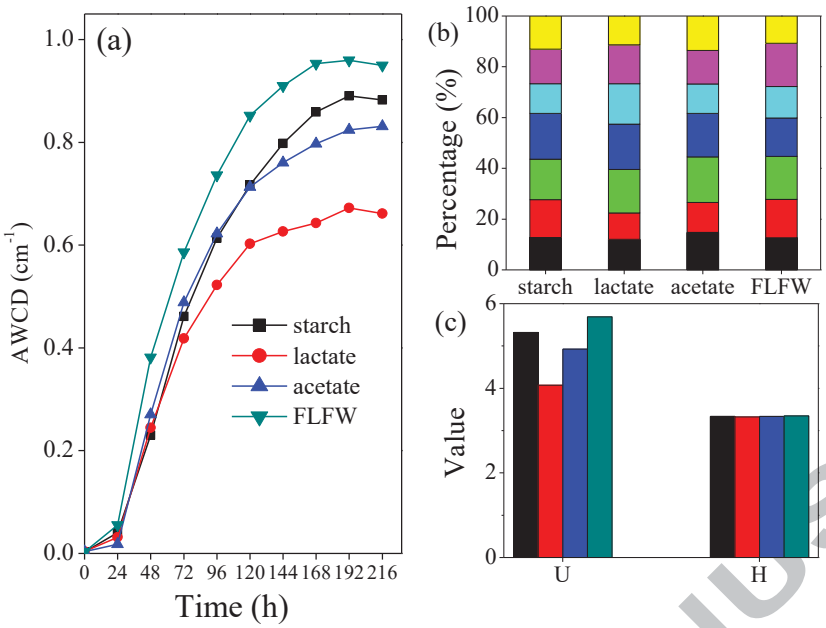

Fig. 4. Carbon source metabolic activity of the activated sludge with the four carbon sources

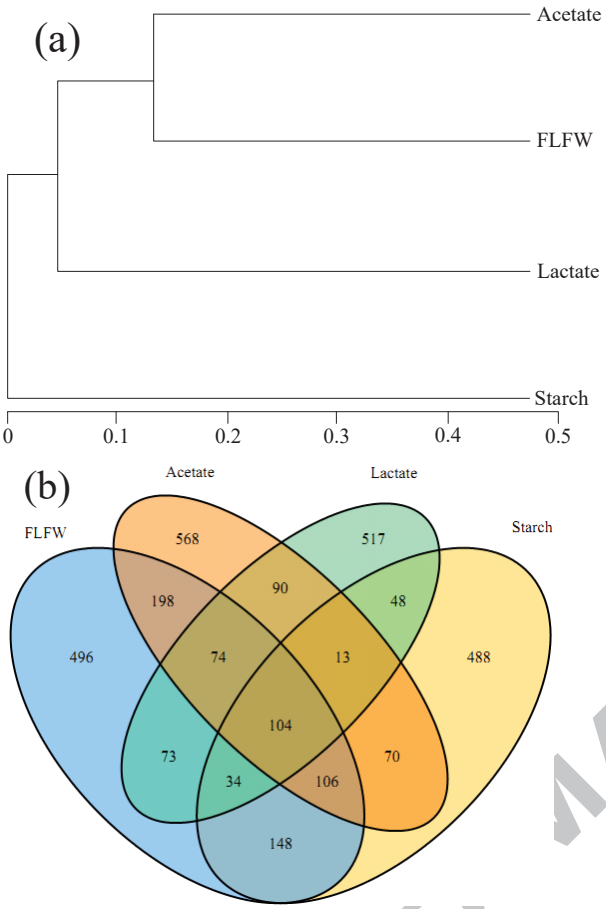

Fig. 5. Bray tree (a) and VEEN diagram (b) based on the OTUs of the activated sludge.

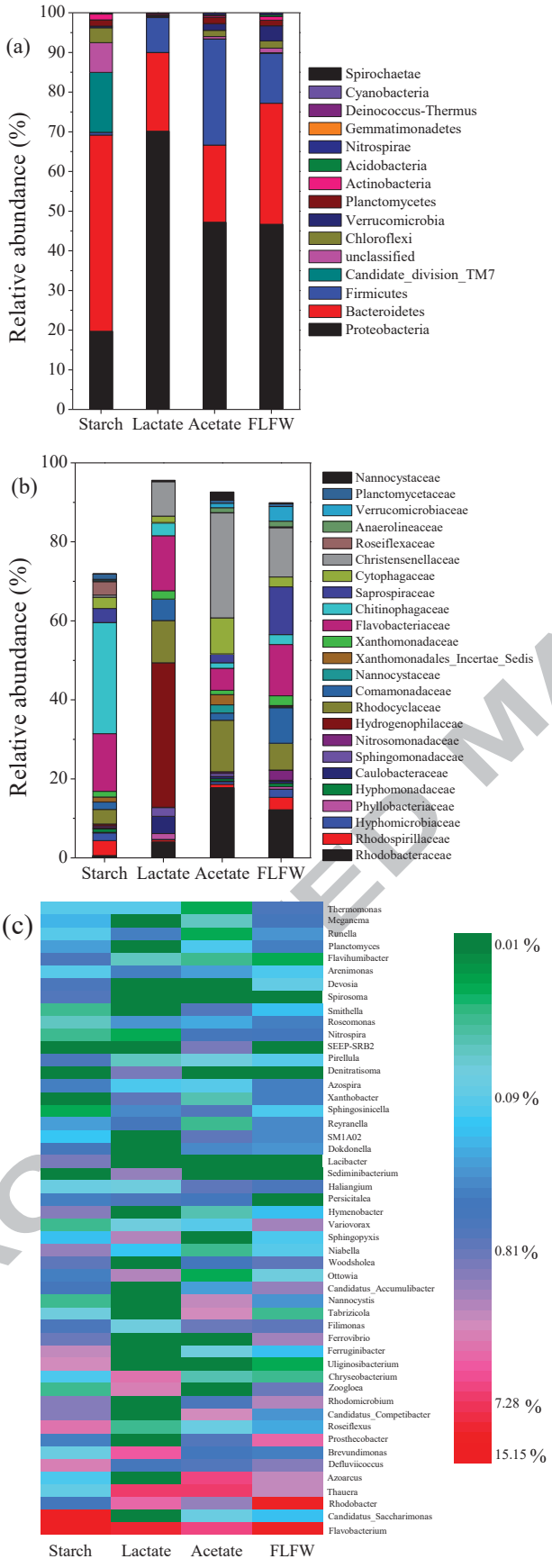

Fig. 6. Taxonomic composition of community structure in the activated sludge. (a) phyla, (b) families with relative abundance >1% and (c) the first 50 genera.