Effects of temperature on lignocellulosic wastes hydrolysis and volatile fatty acids accumulation under neutral and strongly alkaline conditions

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Abstract. The volatile fatty acids (VFAs) produced in the process of wetland plant litter (WPL) anaerobic digestion could be used as external carbon sources to enhance the removal efficiencies of oxidized contaminants in constructed wetlands. In this study, the effects of temperature on WPL hydrolysis and VFAs accumulation under neutral and strongly alkaline conditions were explored. In neutral (pH 7.0) fermentation, biotic factors were the leading reasons for WPL hydrolysis, and the maximal SCOD accumulation (2467 mg L\(^{-1}\)) occurred at 35 °C with a fermentation time of 20 days. In strongly alkaline (pH 12.0) fermentation, abiotic factors were the leading reasons for WPL hydrolysis, and SCOD concentrations increased with temperature at a given fermentation time. Further investigation showed that biotic release of carbohydrate was more sensitive to temperature change than abiotic release. 25 °C was the optimal temperature for biotic release of carbohydrate, while abiotic release of carbohydrate slightly increased with temperature. From the results of linear regression, strong positive correlation was observed between VFAs production and the total release of carbohydrate. The optimal temperatures for VFAs accumulation under neutral and strongly alkaline conditions were respectively 35 and 25 °C, both with a fermentation time of 20 days, and the VFAs concentrations were respectively 1890.1 mg COD L\(^{-1}\) and 1276.4 mg COD L\(^{-1}\). VFAs produced in all fermentations consisted of acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids, with acetic acid being the most prevalent product. The fermentation broth fermented at 35 °C with a fermentation time of 20 days has the highest biological utilizability.

1. Introduction

Oxidized contaminants are frequently found in the effluent of wastewater treatment plants (WWTPs), which pose potential threats to human health and ecological environment. For example, the high concentration of nitrate in water resource may be responsible for methemoglobinemia and diverse kinds of cancers in humans [1]. The existence of sulfate will deteriorate the taste of water and cause laxation and decline of gastric juice acidity in humans [2]. The effluent of WWTPs will also contain disinfection byproducts (DBPs) when chlorine disinfection is practiced. Certain DBPs have toxicity, carcinogenicity and mutagenicity which will pose long-term health risks to the safety of drinking water [3].

As a kind of biological process, constructed wetlands (CWs) are characterized by the advantages of moderate capital costs as well as very low energy consumption and maintenance requirements [4-6], which lead to the high potential for wastewater treatment in developing countries. Among all kinds of CWs, horizontal subsurface-flow constructed wetlands (HSSF CWs) have prevalent anoxic and
anaerobic environment [7], which is beneficial to the reduction of oxidized contaminants. Therefore, HSSF CWs are always set after biological disposal units of WWTPs to enhance the removal efficiencies of oxidized contaminants. However, the majority of the labile organic matters are eliminated via microbial oxidation from the influent, thus it is still difficult to achieve efficient oxidized contaminants removal in most of HSSF CWs.

Compared with traditional carbon sources such as glucose and fructose, WPL has the advantages of low cost, renewable biomass and wide availability [8]. According to Hume et al. [9], the yield of WPL in mature wetland systems can reach 500–2000 mg C m\(^{-2}\) L\(^{-1}\). Chen et al. [10] reported that, VFA is one of the most important products of WPL fermentation which can act as electron donor to enhance the reduction of oxidized contaminants. Therefore, the addition of WPL into HSSF CWs can improve the removal efficiencies of nitrate, sulfate and DBPs [8, 11, 12]. Among many kinds of WPL, Cattail (Typha latifolia) is a rapid-growing aquatic plant that can utilize solar energy effectively which is considered to be suitable for wetlands [13, 14]. At the same time, the main component of cattail is lignocelluloses, so it has a pretty high potential for VFAs production [10].

Anaerobic digestion has the advantages of low cost and high environmental benefits which is considered to be the major way of transforming lignocelluloses into VFAs [15]. Given that initial hydrolysis of particulate organic matters is the rate-limiting step of anaerobic digestion [16, 17], numerous works have been carried on to investigate the methods to enhance the rate of lignocelluloses hydrolysis, such as mechanical methods [18], pyrolysis [19], steam explosion [20] and acid/alkaline hydrolysis [21, 22]. However, very few attempts have been made to investigate the factors which influence the accumulation of VFAs. Our previous work has investigated the effect of pH, in the 6.0–12.0 range, on WPL hydrolysis and VFAs accumulation at ambient temperature. Result demonstrated that abiotic factors were the primary reasons for the improvement in WPL hydrolysis and VFAs accumulation under strongly alkaline conditions while biotic factors dominated the hydrolysis of carbohydrate under neutral and weakly alkaline conditions [10]. However, there are very few studies investigating the combined influences of pH and temperature on WPL fermentation at present. Therefore, in this study, the characteristics of WPL hydrolysis and VFAs accumulation under different temperature and different pH values were investigated.

2. Methods

2.1. Source of WPL, seed microorganism and culture media

In this study, the WPL (cattail, Typha latifolia) was collected from the breeding center of aquatic plants in Xiaoshan district, Hangzhou city, Zhejiang province, in November, 2012. After collection, the cattail litter was cleaned, milled into powders with an average diameter of 0.15mm, and air-dried to a constant mass before finally preserved in a container free from moisture at room temperature (20 °C). The dried powder was then used as the substrate for fermentation. The inoculated sludge was taken from the secondary sedimentation tank of Quyang municipal wastewater treatment plant in Shanghai, China. The culture media was according to the previous literature [8].

2.2. Batch fermentation experiments

Batch fermentation experiments were carried out in 350-mL serum bottles. A total of 216 bottles were prepared (6 temperature conditions × 2 pH conditions × 6 sampling intervals × 3 replicates). These bottles were divided into two groups, with 108 in each, and maintained at pH 7.0 (neutral) and pH 12.0 (strongly alkaline) respectively by adding 2 M sodium hydroxide (NaOH) or 2 M hydrochloric (HCl). The fermentation mixtures in each bottle contained 3 g (dry weight) of raw cattail litter and 50 mL of inoculated sludge with distilled water added to a final volume of 250 mL. For each test, the fermentation bottles were wrapped with tinfoil, capped with perforated screw tops and then sealed with silica grips to ensure that the bottles were airtight. DKY-11 rotary shakers were used to control fermentation temperatures at 10 ± 0.5 °C, 15 ± 0.5 °C, 20 ± 0.5 °C, 25 ± 0.5 °C, 35 ± 0.5 °C and 55 ± 0.5 °C, respectively, while shaking at 150 rpm (rotations per minute).
Previous research has showed that the VFAs concentration could reach to a steady state after 30 d fermentation [23]. Thus the incubations lasted for 30 days and samples of 50 mL were collected every 5 days. The suspensions were centrifuged at 10,000 g for 20 min, after which the supernatants were collected for physiochemical measurement whereas the precipitates were stored at 4 ° C until the polysaccharides were analyzed. In the above fermentation experiment, the source of carbohydrates and VFAs was via the hydrolysis of both WPL and inoculated sludge. In order to rigorously evaluate the effects of pH and temperature on WPL hydrolysis and VFAs accumulation, the contributions of carbohydrates and VFAs produced by the inoculated sludge were excluded through the sludge fermentation test, which was similar to the mixtures fermentation but using inoculated sludge as the sole carbon source.

2.3. Hydrolysis experiments
To help distinguish the contribution of abiotic leaching from microbial processes of WPL hydrolysis, 2 mM NaN3 was added to another 216 bottles preventing the microbial contamination according to Davis et al. [24] and conducted at the same time as the batch fermentation experiments using WPL as the sole carbon source. Every 5 days, the Soluble Chemical Oxygen Demand (SCOD), carbohydrate concentrations were analyzed.

In the batch fermentation experiments, the release of carbohydrate can be obtained through subtraction of carbohydrate content in residual mixtures from the initial mixtures (loss method). However, this method may yield less accurate results if the polysaccharides were insufficient released from the mixtures, especially from the lignocelluloses. Therefore, a method of the selective inhibition of microbial carbohydrate uptake using toluene (3% vol/vol), without affecting the extracellular hydrolysis of the polysaccharides, was applied to direct measure the release of carbohydrate in this study according to Boschker et al. [25].

2.4. Analytical methods
The supernatant described above was filtered through a 0.45-μm polyester film, and then tested for total organic carbon (TOC), SCOD, soluble carbohydrate, protein, as well as VFAs. The analyses of TOC, SCOD, soluble carbohydrate, protein and VFAs were the same as described in the previous publication [8].

3. Results and discussion

3.1. Changes in the composition of WPL after hydrolysis
Cellulose, hemicellulose and lignin accounted for 26.8%, 13.7% and 9.3% of cattail litters respectively in this study. As shown in Table 1, after a 30 days period of fermentation, the extent of lignin removal were significant lower at 10–25 ° C than those at 35 and 55 ° C under neutral conditions. Previous studies had proved that biotic factors are responsible for lignin removal in neutral fermentation[10], while the optimum temperatures for lignin peroxidase, manganese peroxidase and laccase were 39, 39 and 36 ° C [27, 28], which explained the highest removal extent of lignin (40.7%) at 35 ° C. Under strongly alkaline conditions, the removal extent of lignin increased with temperatrue. Because of the solubilization of the lignin in alkaline solutions [29], the removal extent of lignin under strongly alkaline conditions was significant higher than those under neutral condition at the same temperature. The removal extent of hemicellulose increased with temperature under both conditions, and strong alkalinity could also enhance the solubilization of hemicellulose. The highest removal extent of cellulose (60.5%) under neutral condition also appeared at 35 ° C, mainly due to the high activity of cellulase at this temperature as well as the high removal of lignin which limits cellulose hydrolysis by unproductive enzyme adsorption and steric hindrance [30, 31]. Different from lignin and hemicelluloses, the removal extent of cellulose was much lower under strongly alkaline conditions, indicating that the cellulose in cattail litters was mainly α-cellulose, which is refractory in alkaline environment but degradable by bacteria [32].
Table 1. Effect of temperature on removal extent of WPL under neutral (a) and strongly alkaline (b) conditions at the end of the experiment (%).

| Temperature (°C) | pH 7.0 | pH 12.0 | pH 7.0 | pH 12.0 | pH 7.0 | pH 12.0 |
|------------------|--------|---------|--------|---------|--------|---------|
|                  | Cellulose | Hemicelluloses | Lignin |        | Cellulose | Hemicelluloses | Lignin |        |
| 10               | 0       | 0       | 0      | 46.3 ± 2.9 | 0       | 35.7 ± 2.6 |
| 15               | 18.6 ± 2.1 | 0       | 5.0 ± 1.1 | 47.5 ± 2.5 | 2.2 ± 0.4 | 42.9 ± 2.5 |
| 20               | 27.9 ± 2.5 | 8.1 ± 1.2 | 13.9 ± 1.8 | 48.9 ± 3.0 | 5.3 ± 1.5 | 53.1 ± 2.9 |
| 25               | 39.5 ± 2.7 | 10.5 ± 1.4 | 22.0 ± 1.9 | 53.7 ± 3.2 | 17.1 ± 1.8 | 59.4 ± 3.3 |
| 35               | 60.5 ± 3.5 | 15.3 ± 2.0 | 29.0 ± 2.4 | 75.6 ± 4.3 | 40.7 ± 2.7 | 64.3 ± 3.8 |
| 55               | 55.3 ± 2.9 | 20.5 ± 2.2 | 36.1 ± 2.2 | 87.7 ± 5.6 | 32.9 ± 2.1 | 78.6 ± 4.3 |

a Values are means ± SD (n≥3).

3.2. SCOD concentrations

SCOD is a parameter that represents the extent of hydrolysis [10, 33]. As shown in Fig. 1a, in neutral fermentation, the SCOD concentrations at 10 and 15 °C were lower than those at higher fermentation temperatures. In the 20–55 °C range, SCOD concentrations initially reached the peak values and then decreased slowly with time which might be due to the consumption of produced SCOD by methanogens. The maximum values and their corresponding fermentation time at 20–55 °C were as follows: 2152 mg L⁻¹ (20 °C, 25 d), 2254 mg L⁻¹ (25 °C, 25 d), 2467 mg L⁻¹ (35 °C, 20 d), 2208 mg L⁻¹ (55 °C, 30 d). At 35 °C, the highest maximum SCOD concentration could be obtained with the shortest fermentation time, suggesting that the optimal fermentation conditions for organics accumulation in neutral fermentation were 35 °C with a fermentation time of 20 days.

Fig. 1b showed that under strongly alkaline conditions, SCOD concentrations at all temperatures began to maintain relatively stable after 10 days, indicating that WPL hydrolysis was basically completed during the first 10 days of fermentation. This was because that strongly alkaline condition could obviously promote WPL hydrolysis rate and thus shorten the time of organics release. Furthermore, the SCOD concentrations under strongly alkaline conditions were much higher that those under neutral conditions at the same temperature and fermentation time, highlighting the importance of alkaline pH values in converting particulate organics into soluble substances. This was mainly attributable to the toxic effect of stronger alkaline conditions on methanogens and also the severe damage of lignocellulose structure by strongly alkaline conditions. Thus, the results indicated that strongly alkaline conditions could not only promote hydrolysis rate but also improve hydrolysis extent which were consistent with previous researches [10, 34]. Besides, SCOD concentrations increased with increasing temperature at
any given time, suggesting that WPL hydrolysis could be accelerated as temperature increased under strongly alkaline conditions.

3.3. Accumulation of VFAs
As shown in Fig. 2a, under neutral conditions, VFAs concentrations firstly increased, and then decreased with increasing fermentation time at all temperatures, which were similar to the variation tendency of SCOD concentrations in neutral fermentation. VFAs concentrations at 10 and 15 °C were significantly lower than those at other fermentation temperatures at any fermentation time. The maximum values and their corresponding fermentation times at 20–55 °C were as follows: 1789.0 mg COD L⁻¹ (20 °C, 25 d), 1830.0 mg COD L⁻¹ (25 °C, 25 d), 1890.1 mg COD L⁻¹ (35 °C, 20 d), 1880.9 mg COD L⁻¹ (55 °C, 30 d). Apparently, the suitable fermentation conditions for maximal VFAs production were also 35 °C with a fermentation time of 20 days. It was shown from the comparison between Fig. 1a and Fig. 2a that the variation characteristics of SCOD and VFAs concentrations with temperature and fermentation time in neutral fermentation have obvious similarities. They not only had similar variation tendencies but also had the same optimal fermentation conditions, suggesting the close relationship between WPL hydrolysis and subsequent acidogenesis process under neutral conditions.

![Figure 2](image.png)

**Figure 2. Effect of temperature on total VFAs production under neutral (a) and strongly alkaline (b) conditions over time.**

It can be seen from Fig. 2b that VFAs concentrations increased with fermentation time at all temperatures under strongly alkaline conditions. The VFAs concentrations at 10–35 °C had already reached high levels early on the 10th day which accounting 65–86% of maximum value at each temperature. This was due to the enhancement of WPL hydrolysis and organics release caused by strongly alkaline conditions, which provided sufficient substrates for acidogenic bacteria in a short time and thus promoted the accumulation of VFAs. The VFAs accumulation rate at 55 °C was slow and at the same time the VFAs concentrations were lower than those at other fermentation temperatures at any given time, which was not consistent with the relative relationship of SCOD concentrations. This might be due to the inhibiting effects of high temperature and strongly alkaline condition on acidogenic bacteria [35]. Zhang et al. [33] also found that VFAs accumulation was inhibited under high temperature and strongly alkaline conditions. VFAs concentrations under different temperature conditions at any given fermentation time from high to low were VFAs (25 °C) > VFAs (20 °C) > VFAs (15 °C) ≥VFAs (10 °C) > VFAs (35 °C) ≥ VFAs (55 °C). Although the VFAs concentration was still rising after 20 days’ fermentation at 25 °C, its change was not evident, suggesting that the optimal fermentation conditions for VFAs accumulation in strongly alkaline fermentation were 25 °C with a fermentation time of 20 d.

In addition, the average VFAs concentrations under strongly alkaline conditions were higher than those under neutral conditions only at 10 and 15 °C. While at 20–55 °C, the contrary was the case. This was not consistent with the relative relationship of SCOD concentrations.
Temperature and pH value influence not only VFAs cumulant but also VFAs composition by changing microbial community structure and reaction pathways [36]. As shown in Fig. 3a and b, the VFAs consisted of acetic, propionic, iso-butyrinc, n-butyrinc, iso-valeric and n-valeric acids, with acetic acid being the most prevalent product with a fraction of 48–82% and 73–93% in neutral and strongly alkaline fermentation respectively. Compared with strongly alkaline pH value, the percentage of acetic acid was lower at neutral pH value at any temperature in this study. It was due to that some methanogens converted more acetic acids into methane. Propionic acid was the second major VFA with a fraction of 16–36%, and 9–23% in neutral and strongly alkaline fermentation.

3.4. Consumption of carbohydrate

Given that soluble carbohydrate is the main hydrolysis product of lignocelluloses and also an important substrate for VFAs product [37], the biotic and abiotic release of carbohydrate were studied.

As shown in Fig. 4a and b, the total releases of carbohydrate increased firstly and then decreased with increasing temperature which reached the maximum value at 35 and 25 °C under neutral and strongly alkaline conditions respectively. Abiotic release of carbohydrate just had a slight increase as temperature increased, and its variation amplitude with temperature was smaller than that of biotic release of carbohydrate. For example, the increase of temperature from 10 to 55 °C led to only 61% increase in abiotic release of carbohydrate. As for biotic release of carbohydrate, the maximum value at 35 °C was 2140.8 mg COD L⁻¹, a 5.8-fold increase compared to the minimum value at 10 °C. It had the similar condition in strongly alkaline fermentation. This suggested that no matter under neutral or strongly alkaline conditions, the effect of temperature on abiotic release of carbohydrate was not remarkable, and biotic release of carbohydrate was more sensitive to temperature variation. Thus, the major factors that caused the changes of total release of carbohydrate were biotic factors. Furthermore, the biggest contributions of biotic leaching to carbohydrate hydrolysis under neutral and strongly alkaline conditions both appeared at 25 °C, accounting for 67% and 40% respectively, indicating that the optimal temperature for biotic leaching was 25 °C. The contributions of abiotic leaching to carbohydrate hydrolysis were 60–80% under strongly alkaline conditions, which were much higher than those under neutral conditions, suggesting that abiotic leaching was the leading reason for total releases of carbohydrate under strong alkaline conditions.
Figure 4. Effect of temperature on the abiotic release, total release and total consumption of carbohydrate under neutral (a) and strongly alkaline (b) conditions with fermentation time of 20 days.

By comparing the total releases of carbohydrate under different conditions, it showed that the total releases of carbohydrate under strongly alkaline conditions were higher than those under neutral conditions at 10 and 15 °C. As temperature rose, the latter reached the same level with the former at 20 °C, and exceeded the former at 25–55 °C. The weak biotic hydrolysis at 10 and 15 °C under both neutral and strongly alkaline conditions was caused by the inhibitory effect of low temperature. Taking 10 °C condition as an example. The biotic releases of carbohydrate under neutral and strongly alkaline conditions were 396.2 and 343.9 mg COD L\(^{-1}\), accounting for 33% and 20% of total releases of carbohydrate respectively. However, under strongly alkaline condition, the abiotic release of carbohydrate was not significantly influenced by low temperature. Instead, it was improved by alkaline pH and reached 1411.9 mg COD L\(^{-1}\), which was nearly twice that under neutral condition. Thus, the total release of carbohydrate under strongly alkaline condition (1755.8 mg COD L\(^{-1}\)) was higher than that under neutral condition (1126.8 mg COD L\(^{-1}\)). The case at 15 °C was similar. Under neutral conditions, as temperature gradually approached and finally reached the optimal temperature for extracellular enzymes activities, biotic release of carbohydrate gradually increased and finally reached maximum values. Thus the process of hydrolysis and carbohydrate release were dominated by biotic factors at 20–55 °C. While under strongly alkaline conditions, as temperature rose, due to the inhibitory effect of alkaline condition on extracellular enzymes activities, the biotic releases of carbohydrate were much smaller than those under neutral conditions, and at the same time, abiotic release of carbohydrate did not increase markedly. Therefore, the total release of carbohydrate was gradually exceeded by that under neutral conditions.

From the discussion above, although the hydrolysis intensities under strongly alkaline conditions were higher than those under neutral conditions at all temperatures, the releases of carbohydrate were relatively low at temperatures higher than 20 °C. This might explain the less VFAs productions under strongly alkaline conditions in this temperature range. As shown in Fig. 5, a very strong positive correlation (R\(^2\)=0.924) was found between VFAs production and the total release of carbohydrate, suggesting a significant role of carbohydrate release in the subsequent acidogenesis process.

Under strongly alkaline conditions, the consumption rates of carbohydrate (consumption to release ratio) were lower than those under neutral conditions, and showed a decrease from 88% to 64% with temperature rose from 10 to 55 °C. This might be due to the smaller proportions of reducing sugar (i.e. glucose, fructose, xylose and galactose, etc.) in soluble carbohydrate under strongly alkaline and high temperature conditions [10]. Alternatively, reduced PTA, AK (key enzymes related to acetic acid formation) and OAATC, CoA transferase activities (key enzymes related to propionic acid formation) of the cell extracts from alkaline-treated microorganism could also inhibit the consumption of carbohydrate by acidogenic bacteria [37].
3.5. Carbon balance in the liquid

A carbon balance in the fermentation liquor was established to reflect the quality and biological utilisability of carbon source fermented under different conditions. The organic carbon source released in the process of WPL fermentation mainly include soluble carbohydrate and protein, VFAs and some other components which were difficult for microorganism to utilize such as aromatic compounds. As shown in Fig. 6, under neutral conditions, with a fermentation time of 20 days, the percentage of VFAs in total SCOD increased from 14% to 78% with temperature rose from 10 to 55 °C, which showed an obvious change. The percentage of refractory carbon source reached the minimum value at 35 °C, indicating the highest quality of carbon source. Under strongly alkaline conditions, the percentage of VFAs did not have an obvious change with increasing temperature, at the same time, the percentages of refractory carbon source were higher than those under neutral conditions. Therefore, the fermentation broth fermented at 35 °C with a fermentation time of 20 days has the highest biological utilisability.

4. Conclusion

Under neutral conditions, temperature could affect WPL hydrolysis and VFAs accumulation by affecting extracellular enzymes activities and the maximum VFAs production occurred at 35 °C with a fermentation time of 20 days. Under strongly alkaline conditions, WPL hydrolysis efficiency was improved with increasing temperature so that SCOD concentration increased but most of which were components with low bioavailability. The optimal fermentation conditions for VFAs production in strongly alkaline fermentation were 25 °C with a fermentation time of 20 days. The VFAs produced under all conditions consisted of acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids, with acetic and propionic acid being the main products.

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