Effect of carbon source and COD/NO$_3^-$–N ratio on anaerobic simultaneous denitrification and methanogenesis for high-strength wastewater treatment

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The effect of carbon source and COD/NO$_3^-$–N ratio on denitrification and methanogenesis in mixed methanogenic matrix was investigated in this study. Industrial wastewater, anaerobic treated cassava stillage (CS) and glucose synthetic wastewater were used as carbon sources respectively for comparison. Experimental results showed that denitrification was the main nitrate reduction pathway for all COD/NO$_3^-$–N ratios tested in two substrates. Simultaneous denitrification and methanogenesis occurred at COD/NO$_3^-$–N higher than 7 regardless of carbon sources. Incomplete denitrification was observed at COD/NO$_3^-$–N ratio below 7 in both the anaerobic effluent of CS and glucose-fed cultures due to the insufficient available organic carbon. The nature of carbon sources was observed to play a key role in the nitrate and organic carbon utilization rates. COD/NO$_3^-$–N ratio had a strong effect on the organic matter utilization pathways. Methanization consumed more organic matter than denitrification with further increase of COD/NO$_3^-$–N ratio above 7 in two substrates. Results of VFA variation suggested that propionate and butyrate were preferably utilized by the denitrifiers than acetate.

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Nowadays anaerobic digestion process for high-strength industrial wastewater treatment has been intensively studied to remove organic matter and recover renewable energy as biogas at the same time. However, effluents from anaerobic digesters usually contain significant quantities of nitrogen compounds including ammonia which could not be further biodegraded under anaerobic condition (1). The biological denitrification process is considered as a reliable way for nitrogen removal from wastewater, and heterotrophic bacteria use available organic carbon as electron donor to reduce nitrate to nitrogen gas. Although anaerobic effluent contains certain amount of COD, sufficient available organic carbon is still deficient and easily biodegradable COD is usually needed to sustain biological denitrification (2). Simultaneous denitrification and methanogenesis has been observed under anaerobic condition with integrated removal of carbon and nitrogen in a single bioreactor (3–5). The anaerobic digestion intermediates, volatile fatty acids were demonstrated to be a suitable carbon source for denitrification. Complete denitrification was achieved in the anaerobic sequencing batch reactors (ASBR) for piggery wastewater treatment (6). Recently, Ghaniyari-Benis et al. (7) also observed full elimination of nitrate of 3000 mg/L in a multistage anaerobic biofilm reactor at a constant organic loading of 10 g COD/L.d. This process should be an alternative method to the conventional biological denitrification technology, considering its advantage of lower costs of operation of reactors and installations.

In the anaerobic matrix with nitrate amendment, the COD/NO$_3^-$–N ratio and nature of carbon source were found to influence both nitrate reduction pathway and carbon utilization patterns, resulting in the different COD and nitrate removal efficiencies (8,9). The inhibition of the methanogenesis caused by nitrate and/or its denitrification intermediates was noted (15). Using glucose as the only source of organic carbon, Akkuna et al. (8) investigated the influence of carbon to nitrogen ratio on denitrification in anaerobic digesters. Experimental results showed that at COD/NO$_3^-$–N>53, methane production occurred, and ammonification was the main nitrate reduction pathway; simultaneous denitrification and methane production occurred at 8.86≤COD/NO$_3^-$–N≤53 and only denitrification was observed at COD/NO$_3^-$–N<8.86. Moreover, the various carbon sources were also found to be a key factor affecting nitrate and organic carbon utilization pathway. It was reported that nitrate reduction occurred mainly through dissimilatory nitrate reduction to ammonia (DNRA) when using fermentable glucose and glyceral as carbon substrates, whereas denitrification was the predominant nitrate reduction pathway with volatile fatty acids (VFAs) as the carbon source (10). When using sodium acetate as the main carbon source, Ruiz et al. (11) also did not detect significant occurrence of DNRA process even at COD/NO$_3^-$–N of 100. Moreover, Tugas and Pavlostathis (12) reported that the utilization of various electron donors, dextrin/peptone, propionate, acetate and H$_2$/CO$_2$ by nitrate reducers at initial COD/NO$_3^-$–N ratio of 10 had different impact on methanogenesis varying from complete inhibition to full recovery. Thus, the utilization patterns of nitrate and organic matter were strongly dependent on the COD/NO$_3^-$–N ratio and nature of carbon source. However, most
researches have been conducted using pure cultures or synthetic wastewater such as glucose, sucrose, glycerol and volatile fatty acids as electron donors to investigate the effect of C/N and nature of carbon source. Limited researches were reported using industrial wastewater as organic carbon source (1).

In this study, therefore, first stage anaerobic effluents of cassava stillage (CS) and glucose were used as carbon sources respectively to evaluate the effect of nature of carbon sources on denitrifying capacity in mixed methanogenic cultures. The organic carbon and nitrate utilization patterns through denitrification, methanization and DNRA process was studied and compared at different COD/NO₃⁻N ratios in cultures fed with different carbon sources. And the critical COD/NO₃⁻N ratio for simultaneous denitrification and methanogenesis was determined and discussed.

MATERIALS AND METHODS

Inoculum and substrates Anaerobic granular sludge acquired from a full-scale mesophilic upflow anaerobic sludge blanket reactor treating citric acid wastewater was used as inoculum directly without acclimation to nitrate or nitrite. The total suspended solids (TSS) and volatile suspended solids (VSS) of seed sludge were approximately 56.2 and 45.7 g/L, respectively. The anaerobic effluent of CS used as carbon source in this study was obtained directly from the full scale continuous stirred tank reactor (CSTR) which is the first-stage anaerobic digestion treatment process of Taicang cassava ethanol plant (Jiangsu, China). Detailed information of CS can be found in our previous study (20). Table 1 summarizes the characteristics of anaerobic effluent of CS and the residual SCOD concentration was about 4000 mg/L. The anaerobic effluent of CS contained large amounts of VFAs, including acetate, propionate, butyrate and valerate. The synthetic wastewater was prepared with distilled water and contained (in g/L): glucose (4.50); NH₄Cl (0.46); KH₂PO₄ (0.10); NaHCO₃ (3.0) and trace elements (2 mL/L). The trace element solution contained (in g/L): EDTA (5.0); CaCl₂·2H₂O (5.5); FeCl₂·7H₂O (5.5); ZnSO₄·7H₂O (2.2); CoCl₂·6H₂O (1.6); MnCl₂·4H₂O (5.0); CuSO₄·5H₂O (1.6); MgSO₄·7H₂O (5.0); NiCl₂·6H₂O (0.6); Na₂MoO₄·2H₂O (5.0). Both the anaerobic effluent of CS and synthetic glucose wastewater were stored at 4°C before use.

Batch experiments A batch assay was performed to investigate the effect of carbon source and COD/NO₃⁻N ratio on the mixed methanogenic culture in the presence of nitrate. Series of identical borosilicate glass bottles (Witteg, Boro 3.3) were used as reactors with working volume of 400 mL. In each reactor, 100 mL anaerobic granular sludge was added, with 300 mL culture media of different carbon substrates. Two different substrates were used respectively in this assay: anaerobic effluent of CS and synthetic glucose wastewater. The initial COD in each reactor was maintained at 3800 ± 30 mg/L, corresponding to 1138 ± 37 mg TOC/L. Aliquots of NaNO₃ stock solutions were added into each reactor leading to COD/NO₃⁻N ratios of 3, 5, 8, 19, 55 in the anaerobic effluent of CS-fed cultures and 3, 5, 7, 18, 50 in the glucose-fed cultures, respectively. Two reactors were prepared as control cultures without nitrate amendment for comparison purpose. In the anaerobic effluent of CS-fed cultures, the initial pH was 7.80 ± 0.05, while it was adjusted to 7.80 ± 0.05 in the glucose-fed cultures using 5 N NaOH. Then the mixtures in the bottles were flushed with N₂ for 3 min to acquire an anaerobic condition. Incubation of all cultures was conducted at 35°C and rotated in a reciprocating water bath shaker at 110 rpm. The evolved biogas was collected with gas bag and its amount and composition were determined at the same time.

Analytical methods TSS, VSS and pH measurements were conducted according to standard methods (APHA, 1995). The collected liquid samples were centrifuged at 11,000 rpm for 10 min for analysis. The concentration of total organic carbon (TOC) was determined by a TOC analyzer (Shimadzu, TOC-V CPN). Separation and elution of the anions were carried out on IonPac AG11-HC (4 × 50 mm) guard column and IonPac AS11-HC (4 × 250 mm) analytical column utilizing an eluent of 18 mM KOH at an isocratic flow rate of 1.2 mL/min. Cations were analyzed on IonPac CG12A (4 × 50 mm) guard column and IonPac CS12A (4 × 250 mm) analytical column utilizing an eluent of 20 mM methanesulfonic acid at an isocratic flow rate of 1.0 mL/min. Auto suppression mode was used during the detection. Samples for VFA analysis were diluted with a 3% (v/v) H₃PO₄ solution (sample/acid, 1:1 v/v). VFA analysis was done by gas chromatography (Agilent, 6890 N) equipped with a flame ionization detector and analytical column CPWAX2CBP (30 m × 0.25 mm, 0.25 µm). The temperature of the injector and FID were 200°C and 220°C, respectively. Nitrogen was used the carrier gas with a flow rate of 50 mL/min. The GC oven was programmed to begin at 110°C and remained there for 2 min, then increases at a rate of 10°C/min to 220°C, and holds at 220°C for an additional 2 min. The sample injection volume was 1.0 µL. Biogas (N₂, CH₄ and CO₂) composition was determined by gas chromatograph (Agilent, 6890 N) equipped with a thermal conductivity detector (TCD) and analytical column Supelco HayeSep Q (80/100 mesh). The operation temperature at the injection port, the column oven and the detector were 120°C, 35°C and 250°C, respectively. Helium was used as the carrier gas at the flow rate of 20 mL/min.

RESULTS AND DISCUSSION

Nitrate reduction pathway and methanogenic activity at different COD/NO₃⁻N ratios Fig. 1 shows the change of nitrate and nitrite concentration with reaction time in a mixed methanogenic cultures using two different carbon sources, anaerobic effluent of CS (Figs. 1A and B) and glucose synthetic wastewater (Figs. 1C and D) respectively. As shown, nitrate concentration decreased at all tested COD/NO₃⁻N ratios, and different degrees of nitrite accumulation were observed. Moreover, a significant increase of nitrogen gas was detected with nitrate amended cultures, indicating that denitrification process was the main nitrate reduction pathway. When COD/NO₃⁻N ratio was lowered to 3–5, incomplete denitrification was observed in two substrates due to the shortage of available carbon. Significant nitrite accumulation of nearly 145 mg/L was observed in the anaerobic effluent of CS-fed cultures, with about 700 mg/L TOC left in the matrix. While nitrite was completely reduced to nitrogen gas with residual TOC concentration of 87 mg/L in the glucose fed cultures. Sufficient COD should exist in the substrate to ensure complete removal of NO₃⁻–N. The accumulation of denitrification intermediate, nitrite in the anaerobic effluent of CS fed cultures should be attributable to the reason that the portion of the organic matter was not readily degradable compared with glucose, leading to the low denitrification efficiency.

Methane production within reaction time of 19 and 48 h is recorded in Fig. 2. As shown, total amounts of methane production in all the nitrate-amended cultures were lower than those in the nitrate-free cultures. When COD/NO₃⁻N ratio was 3 or 5, no methane was detected even at reaction time of 48 h, indicating that addition of nitrate in such high concentrations resulted in suppression of methane production. Inhibitory effect of denitrification intermediates on methanogenesis might be a significant factor (13–15). Methane recovery was observed at COD/NO₃⁻N of 7–8 in two cultures, and the observed higher methane production in the glucose-fed culture was probably due to the easy utilization of glucose by methanogenesis. In addition, the produced methane increased with the extended reaction time, indicating the occurrence of simultaneous denitrification and methanogenesis in the batch assay. For COD/NO₃⁻N ratio in the range of 7–55, significant methane was detected in two cultures, and more methane amount was detected in the glucose-fed cultures. Inhibition of methanogenesis was reversible and the activity of methanogenesis could be recovered with low initial nitrate concentration, i.e., high COD/NO₃⁻N ratio. Similar results were also obtained in previous studies (15,16). From the foregoing analysis, the COD/NO₃⁻N ratio apparently is critical for the co-occurrence of denitrification and anaerobic methanogenesis.

The variations of aqueous ammonia levels in all cultures versus reaction time are illustrated in Fig. 3. In the anaerobic effluent of CS-fed
cultures, ammonia concentrations in all nitrate amended cultures were lower than the values in the control experiment, further confirming that DNRA process in these cultures did not occur, and denitrification process was the main nitrate reduction pathway. Ammonia concentrations were found to increase in all the nitrate-amended or free, anaerobic effluent of CS-fed cultures after 48 h incubation, probably due to the mineralization of the organic nitrogen in the media. In all the glucose-fed cultures, the ammonia concentration at 48 h was found to be lower than its initial level (Fig. 3). Considering ammonia was the only nitrogen source without other organic nitrogen in the matrix, ammonia nitrogen might be utilized for synthesis of microorganisms in the cultures.

Alkalinity can be generated during the denitrification process, leading to the increase of pH. Therefore, the pH was measured to further characterize the process of denitrification and methanogenesis in the aforementioned matrix. Fig. 4 shows the pH variations in all cultures. The increase of the pH in the nitrate-amended, anaerobic effluent of CS-fed cultures was significant and more than that in the control culture, further confirming the occurrence of denitrification process. Ramakrishnan and Gupta (9) also reported the increased alkalinity in the effluent as a result of denitrification. However, for glucose substrate, it was interesting to notice that the pH decreased to the lowest point at 8 h, and then increased thereafter. Glucose fermentation may occur prior to the denitrification, thus leading to the pH variation.

The foregoing results suggested that regardless of the carbon sources, COD/NO$_3^{-}$-N of 7–8 was the critical ratio for simultaneous occurrence of denitrification and methanogenesis. For a ratio between 7 and 55, denitrification was the main pathway of nitrate reduction, and methanogenic activity was recovered immediately without the presence of nitrate. At ratio below 7, denitrification was the main process and limited by the available organic matter, while the methanogenic activity was inhibited by the residual nitrate. Interestingly, Ramakrishnan and Gupta (9) found that COD/NO$_3^{-}$-N of 6.36 was critical when using phenolic synthetic wastewater as electron donor for denitrification in anaerobic UASB.

**Nitrate and carbon removal at different COD/NO$_3^{-}$-N ratios**

Nitrate and organic carbon removal in 19 h is summarized in Table 2 to further evaluate the effect of COD/NO$_3^{-}$-N ratio and carbon source on denitrification in methanogenic matrix. Nitrate removal efficiency was calculated in terms of NO$_x^{-}$-N (NO$_3^{-}$-N + NO$_2^{-}$-N) considering the detected denitrification intermediate, nitrite. As presented in Table 2, the NO$_x^{-}$-N removal efficiency in 19 h increased with the increase of COD/NO$_3^{-}$-N ratio for two carbon sources, owing to the sufficient carbon concentration for denitrification. The observed lower NO$_x^{-}$-N removal efficiency, corresponding to the lower TOC removal efficiency in 19 h increased with the COD/NO$_3^{-}$-N ratio and carbon source ratios in cultures fed with anaerobic effluent of CS (A, B) and glucose (C, D).

**FIG. 1.** Nitrate reduction and nitrite accumulation and/or reduction at different COD/NO$_3^{-}$-N ratios in cultures fed with anaerobic effluent of CS (A, B) and glucose (C, D).
organic carbon consumption, indicating that the TOC required for the complete nitrate reduction was at least 1.39 mg TOC/mg NO$_3^-$–N, and 1.74 mg TOC/mg NO$_3^-$–N for anaerobic effluent of CS-fed cultures and glucose-fed cultures respectively. At ratio higher than 7 (glucose) and 19 (anaerobic effluent of CS), the methanogenic activity was recovered, and the TOC consumption for per mg NO$_3^-$–N increased significantly, for example, the $\Delta$TOC/$\Delta$NO$_3^-$–N increased to 4.60 and 12.83 at ratio of 18 and 50 respectively in glucose-fed cultures. With the recovery of methanogenic activity, most removed organic carbon was converted to methane. This is agreement with the previous study that the methanization consumed more organic matter than denitrification with further increase of COD/NO$_3^-$–N ratio above 10 (11). Therefore, COD/NO$_3^-$–N ratio had a strong effect on the organic matter utilization pathways.

**Simultaneous denitrification and methanogenesis at critical COD/NO$_3^-$–N ratio** To further understand the organic carbon utilization patterns in simultaneous denitrification and methanogenesis matrix, variations of TOC degradation, NO$_3^-$–N removal and methane production versus reaction time at critical COD/NO$_3^-$–N ratio of 7–8 are illustrated in Figs. 5A and B for anaerobic effluent of CS-fed cultures and glucose-fed cultures respectively. Decreases of TOC concentrations in both control cultures were mainly attributed to methanogenesis, corresponding with the production of methane. Initially the consumption of TOC of nitrate-amended cultures was relatively slow in the first 4 to 5 h, probably due to the acclimation of denitrifiers. With the recovery of denitrifying activity, nitrate concentration was quickly consumed, and the corresponding TOC utilization rate could be calculated from Fig. 5 with the value of 25.5 mg/L·h (19.8 mg/L·h in control) and 55.6 mg/L·h (37.8 mg/L·h in control) for anaerobic effluent of CS fed culture and glucose fed culture respectively. It was obvious that TOC utilization rate through denitrification process is faster than that through methanogenesis. Similar result was also reported by Hendriksen and Ahring (17) and Bernet et al. (18). As shown in Fig. 5, after denitrification process almost completed by 19 h without the presence of nitrate and nitrite, the methane production in the nitrate-amended, glucose-fed culture recovered immediately and its rate was faster than that in the anaerobic effluent of CS cultures. Residual organic carbon was utilized and converted to methane effectively after completion of denitrification process. The performance of simultaneous denitrification and methanogenesis was also closely related to carbon source available in the media. Strangely, a little increase in the TOC concentration was observed at 48 h in the nitrate-amended, anaerobic effluent of CS-fed culture, probably due to further hydrolysis of some colloid organic carbon and the slow recovery of methanogenic activity.

The variations of VFAs at critical COD/NO$_3^-$–N ratio in two carbon source cultures are shown in Fig. 6. With the recovery of denitrifying activity, VFAs (acetate, propionate and butyrate) consumption rates were faster than the values in nitrate free cultures. Propionate and butyrate were found to be preferably utilized by the denitrifiers. Increase of acetate concentration was observed after inhibition of nitrate and nitrite was reduced or eliminated, indicating the recovery
of methanogenic activity. Akunna et al. (19) also revealed that fermentation of glucose in the nitrate-amended culture mainly produced acetate. With acetate, propionate and butyrate as carbon substrates, Hendriksen and Ahring (17) found butyrate and propionate were preferably metabolized by the denitrifiers, leaving acetate for methanogens.

Conclusions

The results of this study showed that COD/NO$_3$--N ratio of 7–8 was the critical ratio for simultaneous occurrence of denitrification and methanogenesis in the anaerobic effluent of CS and glucose-fed culture, regardless of carbon sources. Denitrification process was the main nitrate reduction pathway at COD/NO$_3$--N ratios ranging from 3 to 55. Denitrification was limited by the insufficient available organic carbon at ratio below 7, resulting to the inhibition of methanogenic activity. For ratio higher than 7–8, simultaneous denitrification and methanogenesis was observed and methanogenic activity was recovered immediately after the completion of nitrate reduction. The performance of simultaneous denitrification and methanogenesis was closely related to the nature of carbon sources. Based on the discussion of carbon utilization patterns, denitrification represented the major pathway of organic carbon consumption at low COD/NO$_3$--N ratio. With further increase of COD/NO$_3$--N ratio above 7, the methanization consumed more organic matter than denitrification. COD/NO$_3$--N ratio had a significant effect on the organic carbon utilization pathways. Experimental results on VFAs variation versus reaction time further indicated that the propionate and butyrate were preferably utilized by the denitrifiers than acetate.

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