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Nitrate reduction pathway in an anaerobic acidification reactor and its effect on acid fermentation

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This study investigated the performance of a reactor in which denitrification was integrated into the anaerobic acidogenic process. Industrial wastewater cassava stillage was used as the carbon source, and the nitrate reduction pathway and its effects on acid fermentation were examined. Results from batch and semi-continuous tests showed that the presence of nitrate did not inhibit anaerobic acidification but altered the distribution of volatile fatty acid (VFA) species. Nitrate reduction was attributable to denitrification and to dissimilatory nitrate reduction to ammonia (DNRA). The ratio of DNRA to denitrification was proportional to the ratio of COD/NO_3^-–N. After 130 days of semi-continuous operation, denitrification removal efficiency accounted for about 60% at a COD/NO_3^-–N of 50. The proportional distribution of VFAs was acetate, followed by propionate and then butyrate. The polymerase chain reaction–denaturing gradient gel electrophoresis results confirmed the contributions of denitrification and DNRA in the nitrate-amended reactor and showed that the addition of nitrate enriched the structure of the bacterial community, but did not suppress the activity of acid-producing bacteria.

[**Key words:** Acidogenesis; Denitrification; Dissimilatory nitrate reduction to ammonia; Simultaneous; Microbial community]

Anaerobic digestion processes are widely applied in ethanol wastewater treatment and have become well established. During anaerobic digestion, most of the organic pollutants in the high-strength wastewater can be biodegraded to biogas (CH_4, CO_2). However, the degradation of organic nitrogen results in an effluent containing high levels of ammonia, which is hardly removed under anaerobic conditions and requires further treatment in the follow-up biological nitrification and denitrification processes. In 1994, Akunna and co-workers reported that carbon and nitrate could be simultaneously removed in the anaerobic filter, with direct recirculation of the aerobic effluent (1). Since then, the effect of nitrate and its reduction products on simultaneous denitrification and methanogenesis has been extensively studied (2–4). For example, it was shown that nitrate reduction is superior to methanogenesis in the anaerobic reactor but that nitrogen-containing metabolic compounds could temporarily inhibit methanogenesis. Moreover, the calorific value of the generated biogas is lowered by the presence of nitrogen gas. In attempts to resolve this problem, the simultaneous removal of carbon and nitrogen was investigated, by coupling two-phase acidogenesis and methanogenesis. In those studies, anaerobic activity was not inhibited when the effluxed nitrates were recycled to the acidogenic reactor and complete denitrification occurred (5,6). The nitrate reduction pathway is known to be influenced by the carbon source and by the C/N ratio, either through denitrification or dissimilatory nitrate reduction to ammonia (DNRA). The latter occurs prior to denitrification under conditions of high C/N or easily biodegradable COD, e.g., in wastewater with a high carbohydrate content (7,8). However, since these findings were obtained in studies of simultaneous denitrification and methanogenesis, the roles of the C/N ratio and carbon source in simultaneous denitrification and acidogenesis require further investigation. In addition, synthetic organic wastewater and pure substrates were often used with the aim of investigating the C/N ratio and the nature of the carbon substrates. Consequently there is a lack of experience with practical wastewater.

Therefore in this work, an industrial wastewater, cassava stillage, was used to determine the feasibility of simultaneous denitrification and acidogenesis. Specifically, the effect of the C/N ratio on the nitrate reduction pathway and acid fermentation was examined in batch tests, followed by the realization of simultaneous denitrification and acidification in a single acidogenic bioreactor. The performance of this system and the compositions of the microbial communities were then evaluated.

MATERIALS AND METHODS

Inoculum and substrates Anaerobic granular sludge acquired from the full-scale, mesophilic, anaerobic internal circulation reactor of a paper mill (Jiangsu, China) was used directly as inoculum without acclimation to nitrate or nitrite. Raw cassava stillage (CS) wastewater, obtained as the effluent from a cassava ethanol plant (Jiangsu, China), was chosen because of its high solid organic wastewater content and good biochemical degradability. Table 1 summarizes the characteristics of the CS used in this study. Nitrate was added in the form of sodium nitrate. Both CS and sodium nitrate were stored at 4°C until needed.

Batch experiments The effects of nitrate and COD/NO_3^-–N on acidification and the nitrate reduction pathway were investigated in batch assays. A series of identical serum bottles were used as reactors with a working volume of 500 mL. 50 mL of anaerobic granular sludge and 250 mL of CS were added to each reactor and

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TABLE 1. Characteristics of cassava ethanol wastewater.

Parameter	Average value (mg/L)
Total solids	71500
Volatile solids	58600
Total COD	73200
Soluble COD	40800
Total nitrogen	1700
NH_4^+−N	3.1

then diluted to the desired working volume. The initial pH of each reactor was controlled at 8.00 ± 0.1, using 5 N NaOH and then pH was not adjusted after sampling during batch assays. The bottles were purged with argon for 1 min to avoid residual oxygen interference. The initial total and soluble chemical oxygen demand (TCOD and SCOD) were maintained at around 45,000 mg/L and 21,000 mg/L, respectively. The concentrations of total and volatile solids (TS and VS) in the reactor were, respectively, around 49,100 mg/L and 35,900 mg/L. $NaNO_3$ from stock solutions was added to each reactor to obtain C/N ratios of 30, 41, 77 and 152 in the CS-fed cultures. A control culture without nitrate amendment was also prepared. All cultures were incubated at 35°C in a reciprocating water bath shaker set at 120 rpm. Gas bags were used to collect the evolved biogas and its amount and composition were determined at regular intervals. In addition, 20-mL samples were taken for analysis.

Semi-continuous experiments A semi-continuous assay was performed in serum bottles with a working volume of 210 mL. The hydraulic retention time (HRT) was 3 days. The reactors were operated under a complete mixing condition, and sludge retention time (SRT) was the same as HRT. The initial TCOD was 30,000 mg/L; SCOD ranged from 14,000 to 20,000 mg/L. Aliquots of $NaNO_3$ stock solutions were added to the influent, such that the initial NO_3^-−N concentration was 600 mg/L and the COD/NO_3^-−N ratio was around 50 in the anaerobic CS-fed cultures. A blank reactor of CS alone was prepared for comparison. Every 24 h, 70 mL of the mixture was removed for analysis, substituting the same amount of fresh substrate in the bottles. The initial pH was controlled after sampling at 8.00 ± 0.1, using 5 N NaOH. The final pH of reactors amended with nitrate or not was observed to be 6.6 ± 0.2 and 6.1 ± 0.2 respectively at every 24 h during steady state. The bottles were purged with argon gas for 1 min to maintain anaerobic conditions. The cultures were incubated at 35°C in a reciprocating water bath shaker set at 120 rpm.

In this study, steady state was defined as a sustained concentration of nitrogen compounds within ±10% deviation. During this period, COD, volatile fatty acids (VFAs), and other parameters were determined for ten consecutive days.

Analytical methods Standard methods (9) were used to measure TS, VS, COD, total nitrogen (TN), and NH_4^+−N. The collected liquid samples were centrifuged at 11,000 rpm for 10 min and then filtered through a 0.45-μm filter membrane prior to analysis.

Nitrate and nitrite were analyzed by ion chromatography in a system equipped with two conductivity detectors and two sets of columns (Dionex ICS-3000). Separation and elution of the anions were carried out on an IonPac AG11-HC (4 × 50 mm) guard column and an IonPac AS11-HC (4 × 250 mm) analytical column. The eluent was 18 mM KOH and the isocratic flow rate was 1.2 mL/min. Cations were analyzed on an IonPac CG12A (4 × 50 mm) guard column and an IonPac CS12A (4 × 250 mm) analytical column. The eluent was 20 mM methanesulfonic acid and the isocratic flow rate was 1.0 mL/min. Auto suppression mode was used during the detection.

Samples for VFA determinations were diluted with 3% (v/v) H_3PO_4 (sample/acid, 1:1 v/v) and then analyzed on a gas chromatograph (Agilent, 6890 N) equipped with a flame ionization detector and a CPWAX52CB analytical column (30 m × 0.25 mm × 0.25 μm). The temperature of the injector and detector were 200°C and 220°C, respectively. Nitrogen served as the carrier gas; the flow rate was 50 mL/min. The GC oven was programmed to begin at 110°C and to remain at that temperature for 2 min, followed by increases at a rate of 10°C/min to 220°C, with a final hold at 220°C for an additional 2 min. The sample injection volume was 1.0 μL.

When the TN removal efficiency in the continuous reactor reached steady state, 1.5 ml of mixed culture was withdrawn from the reactors for microbial community analysis. The culture was centrifuged (12,000 ×g, 4°C, 5 min) and the solids were separated. DNA was extracted using the QIAamp DNA stool mini kit (Qiagen, Germany) according to the manufacturer's instructions. The ~170-bp fragments of the V3 region in the 16S rRNA gene of the bacteria were amplified from the extracted DNA by polymerase chain reaction (PCR) with the forward primer F357-GC (5′-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and the reverse primer R518 (5′-ATT ACC GCG GCT GCT GG-3′) (10). The PCR conditions, targeted for bacteria, were as follows: 4 min initial denaturation at 94°C, 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C for 30 cycles, with a final extension for 7 min at 72°C.

Denaturing gradient gel electrophoresis (DGGE) analysis of the PCR amplicons was performed with the Dcode universal mutation detection system (Bio-Rad, USA) on 8% (v/v) polyacrylamide gels in 1× TAE and a denaturant gradient of 30−55%. A 100% denaturing solution consisted of 7 M urea and 40% (v/v) formamide. Same volume of PCR products (nearly 400 ng) was loaded into each gel lane; however, DNA concentration was not controlled in two samples. Electrophoresis was run at 60°C at a constant voltage of 60 V for 16 h. After electrophoresis, the gel was stained with SYBR Green I, photographed under UV transillumination, and documented using the GelDoc system (Bio-Rad).

The target bands were removed from the DGGE gels with sterile pipette tips and placed in sterile vials. DNA was extracted from the DGGE gels with the above-described DNA extraction kit. The extracted DNA was re-amplified using the primers and conditions described above. The PCR products were then sent for sequencing.

Sequencing similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) and used to search the National Centre for Biotechnology Information Sequence Database (http://www.ncbi.nlm.nih.gov/BLAST/).The diversity of the microbial communities was expressed by the Shannon−Wiener index of diversity H'. H' was calculated on the basis of the band intensity on the gel tracks, which was reflected as peak heights in the densitometric curve. The equation for the Shannon index is:

$$H' = -\sum \left(n_i/N\right)\log\left(n_i/N\right) \quad (1)$$

where n_i was the height of the peak and N was the sum of all peak eights of the densitometric curve.

RESULTS AND DISCUSSION

Nitrate-amended acid fermentation and the nitrate reduction pathway in the batch experiments Fig. 1 shows the changes in the VFA profiles with reaction time in CS-fed cultures amended or not with 600 mg NO_3^-−N/L. Initially, the amount of total VFAs in the nitrated-amended culture was similar to that in the nitrate-free culture. After 48 h of fermentation, VFA concentrations were about 50% lower but they progressively increased such that by the end of fermentation they had reached about 13,000 mg/L in both cultures. The main VFA species in the nitrate-free culture after 108 h of fermentation were propionate, acetate, and butyrate, in that order. However, with the addition of nitrate into the system, the butyrate concentration decreased significantly such that the VFAs consisted almost exclusively of acetate and propionate. Compared to the nitrate-free culture, the acetate content increased from 33% to 48%, propionate was present in similar amounts, but butyrate decreased from 17% to 0.04%. This change in the VFA distribution may have reflected carbon source utilization caused by nitrate reduction.

Fig. 2 shows the change in nitrate, nitrite, ammonium, and TN as a function of the reaction time in nitrate-amended cultures. The initial nitrate concentration of around 600 mg-N/L was reduced completely at a reaction time of 60 h. Nitrite, as the metabolic product, initially accumulated but by 96 h was completely degraded. The ammonium concentration increased continuously, reaching 265 mg-N/L after 96 h; by contrast, in the blank control culture the measured ammonium concentration was only 40 mg-N/L after 96 h (Fig. 2A), indicating that nitrate was partially reduced to ammonium and that DNRA had occurred. The change in the TN concentration as a function of the reaction time is further described in Fig. 2B. The significant decrease in TN in the nitrate-amended culture corresponded to a removal efficiency of 38.9% at the end of the reaction, compared to the less than 5% TN removal in the nitrate-free culture. This decrease in TN was due to denitrification. Thus, in this study, the nitrate reduction pathway was completed with denitrification, as the main pathway, but also by DNRA.

The nitrate reduction pathway and VFA production at different COD/NO_3^-−N ratios To further evaluate the effect of the COD/NO_3^-−N ratio on nitrate reduction, the changes in the concentrations of nitrogen compounds at different ratios were determined (Table 2). In the blank reactor, the ammonium concentration did not change significantly whereas in the test reactors it increased, indicative of DNRA. Regardless of the small amount of microbial ammonia assimilation, the decrease in the TN concentration was attributable to denitrification.

As shown in Table 2, the nitrate reduction pathway was sensitive to the COD/NO_3^-−N ratio. As the ratio increased from 30 to 152,