Red mud enhances methanogenesis with the simultaneous improvement of hydrolysis-acidiﬁcation and electrical conductivity

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GRAPHICAL ABSTRACT

ARTICLE INFO

Keywords:
Red mud
Methane
Hydrolysis-acidiﬁcation
Electrical conductivity
Direct electron transfer

ABSTRACT

The role of red mud in the improvement of methanogenesis during sludge anaerobic digestion was innovatively investigated in this study. The results demonstrated that the addition of 20 g/L red mud resulted in a 35.5% increase in methane accumulation. Red mud effectively promoted the hydrolysis-acidiﬁcation of organic compounds in the sludge, which resulted in the increase of protein, polysaccharide, and VFAs by 5.1–94.5%. The activities of key enzymes were improved by 41.4–257.3%. Electrochemical measurements presented direct evidence that the electrical conductivity was signiﬁcantly improved with red mud. More conductive magnetite was formed during the secondary mineralization after Fe(III) reduction by Fe (III)-reducing genes such as Clostridiaceae and Ruminococcaceae. The higher conductivity enhanced the electron transfer between the syntrophic bacteria (Geobacteraceae) and methanogens (Methanosaeta and Methanosarcina), and then improved the methanogenesis. This research provides a novel perspective on the synergism between sludge and red mud for methane production.

1. Introduction

Direct interspecies electron transfer (DIET) is considered as the primary mechanism for interspecies electron exchange, which could be enhanced to improve the anaerobic digestion by the addition of conductive materials (Zhao et al., 2017; Lee et al., 2016). Different conductive carbon carrier materials, including carbon nanotube (Li et al., 2015b), biochar (Yu et al., 2015), carbon cloth/felt (Chen et al., 2014), graphite (Zhao et al., 2015), and granular activated carbon (Liu et al., 2012), and different conductive iron (oxide) carrier materials, including hematite (Kato et al., 2012), magnetite (Zhuang et al., 2015), akaganite (Jiang et al., 2013), and nanoscale zero valent iron (Suanon et al., 2017), helped facilitate DIET between bacteria and methanogens, and then improved methane production. However, either these materials are expensive or the production technologies are complex, which limits their large-scale commercial application.

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http://dx.doi.org/10.1016/j.biortech.2017.08.063
Received 2 June 2017; Received in revised form 8 August 2017; Accepted 9 August 2017
Available online 12 August 2017
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Red mud is a highly alkaline byproduct generated from alumina refining of bauxite ore (Ye et al., 2014), which has led to severe environmental problems, such as groundwater pollution, haze generation, and soil contamination (Salam et al., 2013; Tang et al., 2008). Many efforts have been made to find an environmentally friendly and cost-effective way to utilize red mud, such as in the preparation of coagu- lants, adsorbents and catalysts (Ye et al., 2015; Bento et al., 2016). Iron oxide is typically the major constituent in red mud and has a chemical content ranging from 26.9 wt% to 54.8 wt% (Liu and Li, 2015). The iron oxide in red mud may react as a conductive conduit for DIET in an anaerobic digestion process and improve the electron transfer efficiency (Zhuang et al., 2015).

In addition, hydrolysis-acidiﬁcation is recognized as the limiting step during anaerobic digestion. Certain pretreatments are often employed for the rupture of cell walls, biodegradation of extracellular polymeric substances, and acceleration of the release of soluble substrates for methanogenesis (Li et al., 2016). The high contents of alkaline compounds in red mud such as lime and magnesia may cause a high level of alkalinity, which may be favorable for breaking large organic polymers into smaller molecules and enhancing the efﬁciency of the hydrolysis-acidiﬁcation process (Lin et al., 2009).

However, to our knowledge, there is no related work investigating the response of anaerobic digester to the addition of red mud. Furthermore, although many publications stated that different conductive materials effectively enhanced the anaerobic digestion process by increasing the system conductivity (Yu et al., 2015; Chen et al., 2014), there is not sufﬁcient evidence as few direct electrochemical methods have been utilized to detect the change in a system’s electrochemical properties during/after the addition of conductive materials. Only Li et al. (2015b) employed four-probe electrical conductance measurements to examine the conductivity of sludge in an anaerobic digestion process before and after the addition of single-wall carbon nanotubes. However, the relationship between the performance of the methane production and electrical properties during the anaerobic digestion process was still unclear.

Therefore, this is the first study to elucidate the role of red mud on methanogenesis in a sludge anaerobic digestion system, including methane accumulation, intermediate products and microbial communities. Particularly, various measurements were employed to analyze the change in the electrochemical properties in a complex sludge matrix. This work may provide valuable information to accelerate the anaerobic sludge digestion of with the wastes.

2. Materials and methods

2.1. Materials

Raw Bayer red mud is provided by Shandong Aluminum Industry Corporation (Shandong, China). With the analysis of XRD spectra, it was found that the main mineral compositions of Bayer red mud included hematite (Fe₂O₃, 45.46%), aluminum oxide (Al₂O₃, 17.14%), quartz (SiO₂, 14.22%), rutile (TiO₂, 14.81%), and gibbsite (Al(OH)₃, 5.32%). The Brunner-Emmet-Teller (BET) surface area and total pore volume of red mud were 35.46 m²/g and 0.032 cm³/g, respectively. The inoculum sludge was collected from a laboratory-scale UASB reactor in our laboratory. Waste activated sludge from a local municipal wastewater treatment plant (Fuzhou, China) was used as substrate. Fresh sludge was thickened by gravity settling and then stored at 4 °C to maintain its freshness. The characteristics of the sludge were as follows (average values): total chemical oxygen demand (TCOD) of 30153 ± 548 mg/L; soluble chemical oxygen demand (SCOD) of 1434 ± 56 mg/L; total solids (TS) of 31923 ± 256 mg/L; volatile solids (VS) of 19961 ± 189 mg/L; and pH of 7.24 ± 0.05.

2.2. Anaerobic digestion experiments

Batch experiments were conducted in 250 mL anaerobic serum bottles. A mass of 2.0 g of red mud was added into bottles to construct the red mud supplemented reactors with 10 mL of sludge inoculums and 90 mL of substrate. The bottle without the addition of red mud was designated the “control” reactor. After bubbling the medium and headspace with N₂ gas for 15 min at a rate of 5 mL/min, Teflon®-coated rubber and aluminum crimp caps were used to seal the bottles. Then, the anaerobic digestion reactors were maintained at 35 °C with a constant temperature incubator for a period of 28 days (LRH-1500F, Shanghai Blueprad Instrument Co., China). All experiments were conducted in biological triplicate. Differences were evaluated using student’s t-test, and a p value < 0.01 was considered statistically significant.

The effect of red mud on the hydrolysis-acidiﬁcation of sludge was measured using the same test conditions as the anaerobic digestion experiment for 3 days. However, methanogens were inhibited by BESA (C₅H₄BrO₃SNa, sodium 2-bromoethanesulfonate, Sigma, USA) (Wang et al., 2003). Acetate, propionate, and butyrate were the primary metabolic products and were summed as the VFAs.

2.3. Analytical methods

The analytical methods for total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), total solids (TS), mixed liquor volatile suspended solids (MLVSS), volatile solids (VS) and ammonia were in line with the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). The methane concentration was determined using an Agilent 7890A Gas Chromatograph equipped with a flame ionization detector (FID). The HCl-extractable Fe(II) concentration was measured with the ferrozine technique, as described previously (Lovley and Phillips, 1986). The concentrations of different volatile fatty acids (VFAs) were analyzed using a gas chromatograph (Beifen Co., Ltd. Model, SP-3420) equipped with an FID. The pH was measured with a multimeter (model MultiLine P4, WTW, Germany). The XRD patterns of different sludge samples were detected using an X-ray diffractometer (XRD-6000, Shimadzu, Japan) with Cu Kα radiation at 40 kV and 30 mA and recorded in a 2θ range of 5–70° at a scan speed range of 0.2°/s.

The concentrations of soluble carbohydrate and protein were measured using the anthrone method (Loewus, 1952) and BCA Protein Assay Kit (Thermo Scientiﬁc Pierce), respectively. The equivalent relationships between COD and the substrates were as follows: 1.5 g-COD/g protein, 1.06 g-COD/g carbohydrate, 1.07 g-COD/g acetate, 1.51 g-COD/g propionate, and 1.82 g-COD/g butyrate (Lu et al., 2012). The activities of protease, dehydrogenase, α-glucosidase and alkaline phosphatase were evaluated using the methods reported by Goel et al. (1998). The speciﬁc enzyme activity was deﬁned as a unit of enzyme activity per milligram of MLVSS.

2.4. Electrochemical measurement

Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), scanning electrochemical microscopy (SECM) and chron-oamperometry (CA) measurements were utilized to investigate the electrochemical behaviors of samples in both reactors. The detailed parameters could be found in the supplemental information according to Yu et al. (2015), Yuan et al. (2016) and Koley et al. (2011).

2.5. Microbial community analysis

To understand the change in the microbial communities in different reactors, representative sludge samples were collected and sent to Novogene (Beijing, China) for DNA extraction and amplicon sequencing according to Miseq protocols. The primer pair, 515F–806R, was used as
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protective barrier for the agglomerated microorganisms (Priester et al., results in the increasing content of extracellular polymeric substances COD/L. This is because the harsh environment induced by red mud and the protein concentration from 512.8 ± 10.2 to 538.7 ± 16.3 mg polysaccharide content from 81.8 ± 17.0 to 159.1 ± 22.2 mg COD/L

3.2. Effect of red mud on methane production

The performance of methane production in both anaerobic digestion reactors is shown in Fig. 1. In the initial incubation period of the methanogenic culture (10 days), the methane accumulation in the control reactor was higher than that in the reactor with red mud. However, the subsequent average methane production rate in the reactor with red mud exceeded that of the control reactor. After a period of 28 days, the methane accumulation reached 1.41 ± 0.03 mmol/g VSS in the reactor with red mud, which was 35.5% higher than that in the control reactor.

3.2. Effect of red mud on the hydrolysis-acidiﬁcation process

Compared with the control reactor, adding red mud increased the polysaccharide content from 81.8 ± 17.0 to 159.1 ± 22.2 mg COD/L and the protein concentration from 512.8 ± 10.2 to 538.7 ± 16.3 mg COD/L. This is because the harsh environment induced by red mud results in the increasing content of extracellular polymeric substances (EPS), which can construct the gel-like matrix and then react as a protective barrier for the agglomerated microorganisms (Priester et al., 2006). During the hydrolysis-acidiﬁcation process, part of EPS are degraded after the rupture of cell walls with the high alkalinity of red mud, leading to the release and increase of protein and carbohydrate as the main compositions of EPS.

Many studies have demonstrated that high pH inhibited the activity of acidogenic microorganisms, though it improved the hydrolysis reaction of anaerobic fermentation (Lin et al., 2009). The addition of red mud in this research not only effectively promoted the hydrolysis reaction, but also was suitable for acidogenesis. As the products of hydrolysis-acidiﬁcation, the VFA content in the reactor with red mud also increased by 39.11%, from 469.6 ± 8.6 to 653.3 ± 10.7 mg COD/L, compared with that in the control reactor. Particularly, the content of acetate increased from 95.1 ± 9.4 to 166.7 ± 9.6 mg COD/L after the red mud addition, which might provide a favorable substrate form for methanogenesis. The results were consistent with that reported by Chen et al. (2007), in which the influence of pH on the hydrolysis and acidiﬁcation of sludge was investigated, and found that the hydrolysis and the production of short-chain fatty acids were signiﬁcantly improved under alkaline conditions.

The activities of the key enzymes played an important role during the hydrolysis-acidiﬁcation process. As shown in Table 1, the activities of protease and α-glucosidase increased by 181.9% and 54.2%, respectively, correspondingly leading to the accelerated decomposition of the protein and carbohydrate after red mud addition. Furthermore, alkaline phosphatase has the important physiological role of dephosphorylating compounds, and its activity increased by approximately 41.4% with red mud. Moreover, dehydrogenase has been found to be central to oxidative substrate removal, which is the indicator of the viable biomass fraction (Huang et al., 2008). The activity of dehydrogenase also increased from 0.1462 ± 0.0232 to 0.5224 ± 0.0173 EU/mg MLVSS. These data demonstrated that red mud could signiﬁcantly promote the hydrolysis-acidiﬁcation of sludge, resulting in a 41.4–257.3% increase in the enzyme activities.

3.3. Effect of red mud on the electrochemical properties

CV is a potentiodynamic electrochemical measurement technique that is widely employed to evaluate the electrochemical behaviors of different materials (Zhou et al., 2015). The higher peak current suggests higher redox species, resulting in greater electron transfer performance (Fricke et al., 2008). As shown in Fig. 2A and B, the peak current of both reactors increased as digestion proceeded. More importantly, the sample in the reactor with red mud had higher oxidation and reduction peaks than that in the control reactor at the same period of anaerobic digestion, indicating the different contents of redox-active groups (Okamoto et al., 2014). Red mud effectively promoted the hydrolysisis-acidiﬁcation process, resulting in the increase of redox-active groups such as quinone/hydroquinone groups in humic substances (redox peaks located in the potential range of 0.30 and 0.35 V) (Yang et al., 2014) and cytochrome c in protein (redox peak located in the potential range of 0.10 and 0.15 V) (Dai et al., 2016). Therefore, these results demonstrated that red mud effectively enhanced the electron reduction/oxidation reactions and then decreased the transfer resistance of electrons.

EIS was also used to examine the dielectric properties of the sludge in the anaerobic digestion reactors with and without red mud. It can be seen from Fig. 2C and D that the electrical signature was a circular arc and that the amplitude of this signature gradually decreased in both reactors, suggesting increased conductivity as the anaerobic digestion proceeded. To better reﬂect the change in the electrical properties, modeling an electrical impedance diagram with an appropriate equivalent circuit is necessary. The equivalent circuit in Fig. 2C and D comprised two resistances (R1 and R2) and one capacitor (C1), similar to a Debye equivalent circuit (Frübing, 2011). Ségalen et al. (2015) considered that R2 represented free charges in the material, while C1 and R1 were related to the solid network consisting of polymeric substances such as bound charges. The R2 values of both reactors decreased with digestion time. Meanwhile, the R2 value in the reactor with red mud was lower than that in the control reactor. When the anaerobic digestion process ended, the R2 values of the reactors with and without red mud were 253 and 308 Ω, respectively. These results also implied that the addition of red mud effectively improved the conductivity of the anaerobic digestion system and correspondingly improved the transfer of free charge.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Protease</th>
<th>α-Glucosidase</th>
<th>Alkaline phosphatase</th>
<th>Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.60 ± 0.08</td>
<td>1.42 ± 0.05</td>
<td>12.48 ± 1.32</td>
<td>14.62 ± 2.32</td>
</tr>
<tr>
<td>Red mud</td>
<td>4.51 ± 0.11</td>
<td>2.19 ± 0.12</td>
<td>17.65 ± 0.85</td>
<td>52.24 ± 1.73</td>
</tr>
</tbody>
</table>

Table 1: Specific activities of key enzymes after the fermentation for 3 days (10⁻² EU/mg MLVSS).

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a universal gene primer to amplify the V4 region of the 16S rRNA gene with the barcode. The detailed sequencing process is shown in the supplemental information.

3. Results and discussion

3.1. Effect of red mud on methane production

3.2. Effect of red mud on the hydrolysis-acidiﬁcation process

Fig. 1. The change of methane accumulation in the control reactor and the reactor with red mud. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Sludge is more conductive as the anaerobic digestion proceeded. Strong relationships are observed between the methane production and electrical properties. The coefficients of determination ($R^2$) between the resistance $R_2$ and the methane accumulation in the reactors with and without red mud were 0.9471 and 0.9243, respectively, which illustrated that $R_2$ was proportional to the methane production.

SECM can effectively quantify the primary metabolite of the substrates in the x-y direction, providing a spatial concentration profile over the surface (Kanno et al., 2015). Fig. 3 shows the SECM topographic images of different samples, which exhibited obvious topographic changes. More peaks of high current appeared as the digestion proceeded, suggesting a higher concentration of redox-active or conductive small molecules on the micrometer scale (Koley et al., 2011). In addition, the reactor with red mud had a higher current than the control reactor. When the process ended, the maximum current in the reactor with red mud reached 0.33 $\mu$A, which was higher than that in the control reactor (0.25 $\mu$A). These data validated that the high content of conductive materials in red mud such as hematite played an
important role during the anaerobic digestion process, which effectively improved the conductivity of the anaerobic digestion system. In addition, it may be also due to the difference of composition and molecular size of compounds in the system (Dieudé-Fauvel et al., 2014). More small size compounds with high mobility were produced during the hydrolysis-acidification process with red mud addition, which also improved the electrical properties of the anaerobic digestion system (Liao et al., 2002). The conclusion is agreement with the CV and EIS results.

The decomposition of organic materials is often coupled with Fe(III) reduction (\(\text{CH}_3\text{COO}^- + 8\text{Fe(III)}(s) + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 9\text{H}^+ + 8\text{Fe(II)}(l)\)) (Ottow, 1970). During the anaerobic digestion process in this research, the Fe(II) concentration in the control reactor fluctuated slightly. In contrast, the Fe(II) concentration in the reactor with red mud first significantly increased and then decreased. The highest Fe(II) concentration was 1.51 ± 0.02 mmol (Fig. 4A). XRD spectra of samples from the reactor with red mud addition at 0, 7, 14, 21, and 28 days demonstrated the occurrence of secondary mineralization, which led to a decrease in the Fe(II) concentration and the formation of more conductive magenite. The results were in accordance with those reported by Jiang et al. (2013), who also reported the transformation of Fe(III)/Fe(II) during anaerobic digestion.

To further verify the electron-accepting capacity of red mud, the redox property of red mud was evaluated through a mediated electrochemical analysis. It can be seen from Fig. 4B that each spike of 50 mg of red mud resulted in an obvious peak current isolated from the background current, indicating the existence of surface redox-active moieties, such as structural Fe, in red mud. The electron-accepting capacity (Q_{EAC}) and electron-doating capacity (Q_{EAD}) were 0.4217 ± 0.0273 μmol e⁻/g red mud and 0.0023 ± 0.0001 μmol e⁻/g red mud, respectively. The high Q_{EAC} value indicated that most of the surface redox-active moieties of red mud were in oxidized forms that could accept electrons. Previous studies have demonstrated that Fe(III) reducing microorganisms had superiority over methanogens in competing with the electron donors (Hori et al., 2010). This meant that the Fe(III) reduction was a more favorable electron sink for the acetate oxidation at the initial stage of anaerobic digestion in the reactor with red mud, which led to a lower methane accumulation compared with the control reactor.

### 3.4. Effect of red mud on the microbial community

After 28 days, Illumina Miseq sequencing targeting the V4 regions of the 16S rRNA gene was employed to characterize the change in the microbial community in both reactors. It was found that *Syntrophorhabdaceae* and *Syntrophomonadaceae* were detected in both reactors, which can metabolize organic acids to acetate with the production of H₂ in co-culture with H₂-consuming methanogens (Sousa et al., 2007). Their relative abundance was higher in control reactor than that in the reactor with red mud addition, indicating the more important role of H₂-consuming methanogens in control reactor. In contrast, the addition of red mud resulted in the enrichment of *Clostridiales* and *Ruminococcaceae* from 21.55% and 10.36% to 27.79% and 15.54%, respectively. The main genus in the two families was close to *Clostridium* species, which belong to the Fe (III)-reducing genes and had the type IV pili for extracellular electron transfer (Bordeleau et al., 2015). Meanwhile, the abundance of *Synergistaceae* was also increased with red mud addition. It was in line with the results reported by Zhao et al. (2017), in which *Synergistaceae* was enriched with the addition of granular activated carbon and was speculated to be able to transfer electrons to Fe(III) oxides. Notably, *Geobacteraceae* with the abundance of 1.18% was detected in the reactor with red mud addition, while no *Geobacteraceae* was found in the control reactor. The potential of Fe (III) reduction by *Geobacteraceae* was demonstrated by previous research (Lovley and Phillips, 1988). These data indicated that red mud could enrich Fe(III)-reducing genus through the dissimilatory iron reduction.

The differences in the methanogenic communities between two reactors were investigated, and the results demonstrated that *Methanobacterium* and *Methanoseta* constituted the primary abundant methanogens in both reactors. *Methanobacterium* species were hydro-gen-utilizing methanogens, and its relative abundance in the control reactor was higher than that in the reactor with red mud addition. In contrast, the abundance of *Methanoseta* species increased from 38.85% to 62.90% after the addition of red mud. In addition, *Methanosarcina* also slightly enriched with red mud.

Syntrophic organisms play a key role in the DIET-mediated methanogenic process. *Geobacter* species can not only oxidize diverse organic compounds by reducing metals, but also transfer electrons extracellularly. The enrichment of *Geobacteraceae* (Geobacter sulfurreducens) after the addition of red mud indicated that red mud was reacted as a terminal electron acceptor during the acetate oxidation. Kato et al. (2012) and Lee et al. (2016) also found the enrichment of *Geobacter* species with the supplement of conductive particles. Moreover, electron-accepting methanogenic archaea are also necessary during the DIET process (Li et al., 2015a). In this study, the relatively abundance of *Methanoseta* and *Methanosarcina* was higher in the reactor with red mud than that in the control reactor, which possessed the ability to accept electrons directly. The presence of conductive materials in the red mud, such as hematite and magnetite, effectively strengthened the associations between the syntrophic bacteria (*Geobacteraceae*) and methanogens (*Methanoseta* and *Methanosarcina*), which triggered the electron transfer and the CO₂ reduction to CH₄ by methanogenesis (\(\text{CO}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}\)). The results
were consistent with the improved performance of methane production after the addition of red mud.

During the anaerobic digestion process, methanogens are more vulnerable to ammonia (ammonium ion (NH₄⁺) and free ammonia (NH₃)) compared to other groups of microorganisms (Wang et al., 2016). The inhibition of ammonia on the anaerobic digestion process occurs when the ammonia concentration is higher than 1500 mg N/L (Zhang and Angelidaki, 2015a, 2015b). Although the alkaline compounds in red mud promoted the hydrolysis of sludge flocs and the conversion of protein to ammonia, the ammonia concentration with red mud addition was 48–635 mg N/L in this research, which should not inhibit the anaerobic digestion process. Therefore, as shown in Fig. 5, the important role of red mud in enhancing the methane production during sludge anaerobic digestion includes two pathways: first, red mud promoted the hydrolysis-acidification of the sludge, which resulted in a higher content of substrates and enzyme activities; second, red mud served as an effective mediator for electron transfer during methanogenesis after the Fe(III) reduction.

4. Conclusion

The results demonstrated that red mud effectively enhanced methanogenesis, increasing methane production by 35.5% compared with the control reactor. This is partly because red mud promoted the hydrolysis-acidification of the sludge, which resulted in an increase in substrates and enzyme activities. In addition, CV, EIS and SECM testing implied that the addition of red mud significantly improved the electrical conductivity, which strengthened the DIET between syntrophic bacteria (Geobacteraceae) and methanogens (Methanoseta and Methanosarcina), and enhanced the reduction of CO₂ to CH₄ by methanogenesis.

Acknowledgements

The authors are thankful for grants from the National Natural Science Foundation of China (51608121, 41671264, 41601241), the Project of Fujian Provincial Department of Science and Technology, China (2016NS004), and the Project of Fujian Development and Reform Commission, China (20160527) that supported this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.08.063.

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