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Research article

Deep exploitation of refractory organics in anaerobic dynamic membrane bioreactor for volatile fatty acids production from sludge fermentation: Performance and effect of protease catalysis

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ABSTRACT

Volatile fatty acids (VFAs) production from waste activated sludge fermentation could be improved in anaerobic dynamic membrane bioreactor (ADMBR) by retaining residual organics within the reactor and prolonging their reaction time. However, the accumulation of refractory organics made it operate unstably. Therefore, protease catalysis was adopted to deeply exploit those refractory organics in sludge. By combining with dynamic membrane retention, protease catalysis indeed presented a good performance. VFAs yield was further enhanced by over 40% in ADMBR. Membrane fouling was slightly relieved due to protein and polysaccharide degradations in the sludge of dynamic membrane. It was also interestingly found that not only protease activity of sludge was improved from 5 to 21 U/ml, but also β-GLC activity was enhanced from 13 to 20 μmoL/L/h. Microbial community analysis showed protease addition could reduce bacterial richness and evenness in sludge, and accelerate the growth of polysaccharides-hydrolyzing bacteria, as well as inhibit some proteolytic bacteria.

1. Introduction

In recent years, the amount of waste activated sludge (WAS) has been increasing in wastewater treatment plants (WWTPs) of China, and reached even 55 million tons with water content of 80% in 2015 (National Bureau of Statistics of People's Republic of China, 2016). Anaerobic fermentation for volatile fatty acids (VFAs) production provides a new alternative for WAS reduction and resource recovery. Especially, with the introduction of "carboxylate platform" (Agler et al., 2011), technologies of efficiently utilization of VFAs from sludge fermentation, such as high-added value long-chain fatty acids (Li et al., 2018; Cavalcante et al., 2017; Zhang et al., 2017) and fatty acid methyl ester (Wallis et al., 2017), have greatly stimulated the vigorous development of WAS fermentation for VFAs production.

Low conversion rate of organics in WAS, mainly caused by the residuals of proteins and polysaccharides, is the bottleneck restricting the application of fermentative VFAs production technology (Liu et al., 2013; He et al., 2018). About 60–70% of organic matters in WAS has the potential of being biologically utilized (Hao et al., 2014) while only 30–40% of them is finally converted into VFAs (Yin et al., 2016). Moreover, the organics remained in the residual sludge not only leads to the low efficiency of sludge anaerobic fermentation, but also influences the dewatering performance of the fermented sludge (Zhu et al., 2015). The introduction of dynamic membrane separation into conventional sludge fermenter could greatly improve the conversion efficiency of organics in sludge by prolonging substrates retention time and relieving products feedback inhibitions (Liu et al., 2016a). However, long-term operation showed that there were still a large amount of organic residues, especially refractory protein, accumulated in the ADMBR, which made its operation instable.

In order to improve the conversion rate of organics in WAS, previous attentions mainly focused on sludge pretreatment, but neglected the exploitation of those organic residues. However, by flask tests, Yin et al. (2016) proved those organic residues were indeed exploitable. There are already many kinds of methods for the improvement of organics conversion in WAS, such as thermal, alkaline, ultrasonic and oxidative hydrolyses (Han et al., 2017; Xiao

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et al., 2015; Jochen et al., 2018; Marta et al., 2007), but most of which would cause serious lysis of functional bacteria and are not suitable for the on-site application in biological reactors. It was reported that biological enzyme hydrolysis had the characteristics of mild reaction condition, few by-products, low energy consumption and high efficiency during sludge pretreatment (Barjenbruch and Kopplow, 2003; Yu et al., 2013; Kim et al., 2013). Therefore, though there is no related reports yet, protease catalysis seems to be suitable for the online exploitation of the accumulated residuals in ADMBR.

Furthermore, by comparing with conventional anaerobic bioreactors, there are several superiorities that make protease catalysis more suitable to be applied on ADMBR. Firstly, refractory organics can be concentrated within reactor by the retention of dynamic membrane, which greatly improves the efficiency of protease catalysis and reduces the amount of protease consumption. Secondly, most of the externally added protease can be retained in the reactor by membrane separation, especially for those combining with macromolecule organics, which makes the added protease fully utilized and provides the possibility of stable operation for ADMBR. Finally, under the pressure of sludge cells disruption due to protease catalysis, membrane separation could help non-sensitive bacteria, such as bacteria with cuticle, to survive and flourish. In conventional reactors, those bacteria could be washed out along the discharging due to their slow growth rate.

Thus, the objective of this study is to accelerate the hydrolysis of the residual organics in ADMBR by on-site application of protease catalysis, and ensure its stable operation in aspects of VFAs production, membrane fouling and microbial maintain. The performance of protease catalysis in promoting VFA production and residual organics degradation were investigated, and its influences on membrane fouling and microbial hydrolase activities were analyzed. The sustainability of microbial ecosystems was evaluated according to diversity indices, taxonomic distribution and redundancy analysis (RDA).

2. Material and methods

2.1. ADMBR

The ADMBR used in this test was the same as the reactor reported in previous report (Liu et al., 2016a). The operation conditions were also adopted the optimal values obtained in previous experiments, including total hydraulic reaction time (HRT) of approximately 5.4 d (Ma et al., 2016), pH of around 10.0 (Liu et al., 2012; Feng et al., 2009) and normal temperature of about 37 °C. Also, dynamic membrane subassembly was used in the ADMBR, in which, silk with an aperture of approximately 75 μm was used as the separation layer. The long HRT made a low membrane flux of 1.0–1.5 ml/m^2·min feasible in the ADMBR, which greatly relieved membrane fouling. After sampling on the 8th day, the influent and effluent were stopped for 8 h, but the continuous stirring was kept to maintain stable cross-flow on membrane surface for online cleaning dynamic membrane. Then, the ADMBR was operated normally. Therefore, there are about 16 h for the re-forming of dynamic membrane before sampling on day 9, during this period, the missed feeding was replenished to keep the organic loading constant. Then, protease was added along with feeding on day 10.

2.2. Substrates

WAS used as the substrate in this study was sampled, pretreated and stored just as the same as that mentioned in the previous paper (Liu et al., 2016a). The WAS had a pH of 6.7 ± 0.3, a soluble COD (SCOD) of 0.9 ± 0.08 g/L, a soluble VFAs concentration of 0.75 ± 0.06 g/L, a total solids (TS) concentration of 15.3 ± 1.3 g/L, a volatile solids (VS) concentration of 6.3 ± 0.4 g/L, a soluble protein concentration of 1.1 ± 0.1 g/L, and a soluble reducing sugar concentration of 0.4 ± 0.04 g/L.

2.3. Seeding sludge for anaerobic fermentation

Fermented sludge from another stably operating ADMBR for sludge fermentation was collected as the seeding sludge in this test. It had a pH of 10.5 ± 0.3, a SCOD of 10.25 ± 0.15 g/L, a soluble VFAs concentration of 4.5 ± 0.4 g/L, a TS concentration of 15.25 ± 0.3 g/L, a VS concentration of 4.0 ± 1.0 g/L, a soluble protein concentration of 0.85 ± 0.25 g/L, and a soluble reducing sugar concentration of 0.55 ± 0.03 g/L.

2.4. Hydrolase

Hydrolase used in this test was commercial alkaline protease with activity of 80000 U/g (from Amano in Japan), and could kept activity in the pH range of 9–12. The theoretical value of microorganism protease activity in sludge contributed by externally protease addition (μ, U/ml) could be calculated out by the model 1.

$$\mu = D \times U \times TS \times 10^{-6} \quad (1)$$

where D is the addition dosage of the commercial alkaline protease (mg protease/g WAS), U is the activity of the commercial alkaline protease (U/g), and TS is the total solids in ADMBR (g/L).

2.5. Anaerobic fermentation

As enough reports had proved that alkaline fermentation of WAS presented higher VFAs production than those of neutral and acidic fermentations (Li et al., 2017; Zhao et al., 2018; Ma et al., 2016), WAS alkaline fermentation with pH of 10 was thus adopted in this study. The total process of sludge anaerobic fermentation could be divided into two stages, without (stage 1) and with (stage 2) protease addition. In stage 1, the fermenter was filled with WAS of 13 L and seeding sludge of 1.3 L. Dissolved oxygen in the WAS and the gas in the headspace of the flasks were removed by sparging gaseous nitrogen for approximately 30 min to maintain a strictly anaerobic condition. Then, pH was adjusted to 10.0, temperature was controlled at about 37 °C and stirring intensity was stabled at 100 r/min. During the period of batch operation in day 1–3, full effluent recycle was implemented to accelerate dynamic membrane formation, that is, all of the effluent was pumped into reactor again as the influent. As the experiment proceeded, VFAs concentration in fermenter increased. When the VFA concentration in reactor tended to be stable, continuous feeding and discharging were implemented. Then, keeping stable operation until that the VFA concentration in the effluent reached stable, stage 1 ended and stage 2 began. In stage 2, operation parameters of TS, temperature, pH, HRT and stirring intensity, were kept constantly, while protease with dosage of 25 mg/g dry sludge (DS) was added along with the feeding. The protease dosages of 0, 5, 10, 15, 20, 25 and 30 mg/g DS had been tested by beaker experiments and 25 mg/g DS was found to be the optimum. Samples were removed from fermenter and analyzed at intervals of 24 h.

2.6. Analytical methods

2.6.1. Measurements of conventional indexes

Conventional indices, including pH, COD, VS, TS and VFA, as well as soluble carbohydrate and protein concentrations were measured according to those methods mentioned in previous paper (Liu et al.,

2016a). VFA value was converted to COD and VFA concentration was recorded in the form of COD concentration. Organic chemistry groups on the surface of dynamic membrane were determined by Fourier transform infrared spectroscopy (FTIR, Tensor 27, Bruker Optics, Germany). All of the measuring experiments were conducted independently in triplicate and parallel values were treated by variance analysis.

2.6.2. Measurements of enzymatic activities

β-Glucosidase (β-GLC) activity, representing the capability of polysaccharide degradation, was measured spectrophotometrically (Mapada UV-1600, Shanghai, China) in 5-cm cuvettes according to the procedure published by Li and Chróst (2006). Protease activity was determined by the standard method (SB/T, 1988).

2.6.3. Microbial community analysis

The microbial detection and analysis were performed according to the methods published by previous paper (Liu et al., 2016a). Each sample was analyzed in triplicate, and the standard deviations of all analyses were always less than 5%.

3. Results and discussion

3.1. Performance of protease catalysis in improvement of VFA production

Protease catalysis could greatly improve VFAs production from sludge anaerobic fermentation in ADMBR. As shown in Fig. 1A, without protease addition, the concentration of VFAs reached 1.95 g/L in the reactor of ADMBR on the 4th day and tended to be stable at 2.18 g/L on the 8th day. After addition of protease on the 10th day, the VFAs concentration quickly increased again and reached 3.25 g/L in the reactor on the 19th day from 2.25 g/L on the 10th day. Although a part of the produced VFAs was retained in the reactor due to the compact structure of the dynamic membrane in later period of the experiment, VFAs concentration in effluent also increased from 1.91 g/L to 2.70 g/L. Obviously, there exits synergistic effects between dynamic membrane retention and protease catalysis. Though most of the residual protein could be retained by dynamic membrane in ADMBR (Liu et al., 2016a), a large part of which was actually low-biodegradable (Yin et al., 2016) and difficult to be directly conversed for VFAs production. Protease hydrolysis was ever proved to be able to make those refractory proteins degradable either in pre- or post-treatment processes (Liu et al., 2016b; Yin et al., 2016). In this study, results indicate the terminated fermentation of those intercepted refractory proteins can be directly re-activated again by protease catalysis during fermentation process, and the yield of VFAs can be further enhanced by more than 40% in ADMBR.

Moreover, protease catalysis could further accelerate selectively acetic acid production in ADMBR. As shown in Fig. 1B, acetic acid is the dominant component in whole test process, possibly due to the high pH of approximately 10 adopted in this test (Yuan et al., 2015). However, acetic acid proportion in the total VFAs could be further increased from 50% to 65% after protease addition. Previous studies showed that acetogens with cuticle could maintain competitive advantages in severe environments (e.g., alkaline condition) (Liu et al., 2012). This situation for acetogens should become more prominent after protease catalysis adopted in ADMBR, resulting in the enhanced proportion of acetic acid.

3.2. Deep exploitation of the substrates in sludge by protease catalysis

Application of dynamic membrane in sludge fermenter could greatly improve VFA production by the retention to substrates and prolonging their degradation time (Liu et al., 2016a). However, there still exists two problems need to be solved in ADMBR: 1) about half protein in sludge was not conversed into VFAs and lost along with the effluent. 2) the accumulation of refractory protein resulted in the continuous increase of protein concentration in reactor, which would hinder the long-term operation of ADMBR, such as fastening membrane fouling.

Obviously, protease catalysis could greatly accelerate protein degradation in ADMBR. As shown in Fig. 2A, without protease addition, the concentration of protein in reactor always kept at high level and gradually increased from 980 to 1200 mg/L within 7 days, and a large part of protein, as high as 700 mg/L, was lost in the effluent. After adding protease, protein concentrations both in reactor and effluent sharply reduced, and reached 600 and 150 mg/L within about 8 days, respectively. Therefore, results indicate protease catalysis could not only promote refractory proteins decomposition, but also improve the hydrolysis of easily biodegradable proteins.

However, as shown in Fig. 2B, it was interestingly found that protease catalysis could lead to the increases of polysaccharide concentrations both in reactor and effluent. Firstly, the same as the changes of protein concentration, polysaccharide concentrations were also gradually improved by the retention of dynamic membrane when protease was not added into ADMBR. Then, with

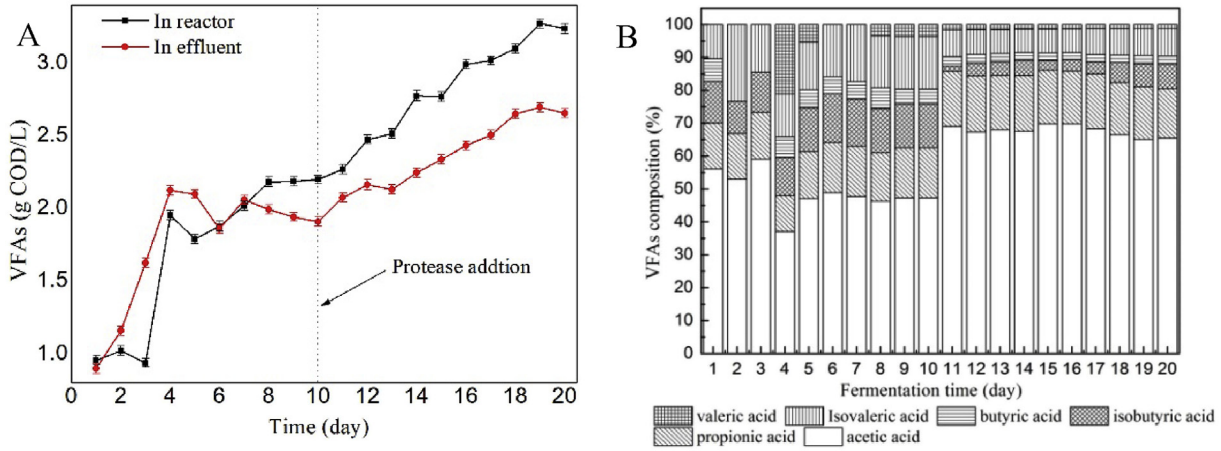

Fig. 1. Influence of protease catalysis on VFAs production in ADMBR.

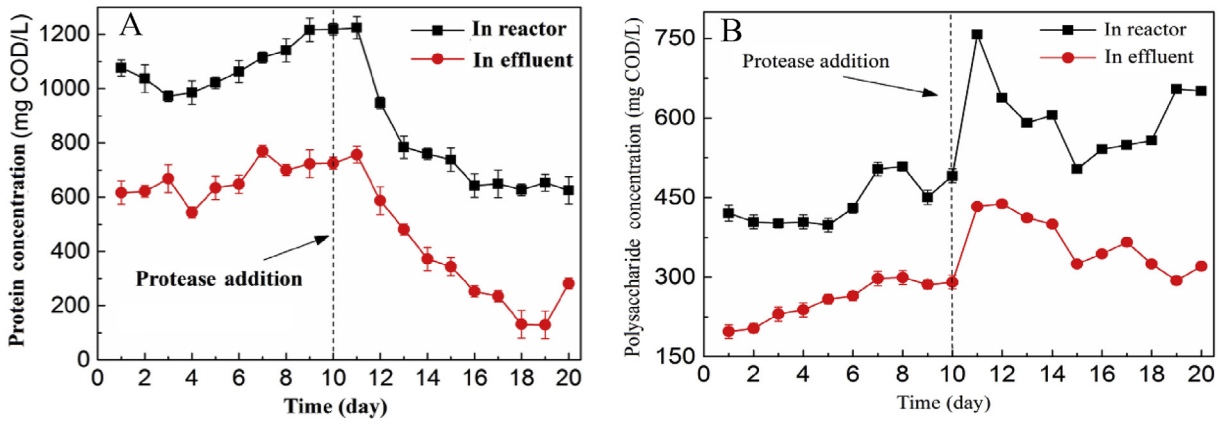

Fig. 2. Influence of protease catalysis on protein and polysaccharide degradations.

protease addition, polysaccharide concentrations did not reduce like that of protein concentration, but sharply increased both in reactor and effluent, though the former fluctuated greatly and the later reduced gradually in the following operation. The rapid decomposition of glycoprotein induced by protease catalysis should be the main reason to the sharply increasing of polysaccharide concentration (Le-Clech et al., 2006).

3.3. Alleviation of dynamic membrane fouling by protease catalysis

Previous studies showed that protein content in sludge was one of the most important impacts on membrane fouling, and larger value of protein/polysaccharide often had more serious membrane fouling (Liu et al., 2018; Ao et al., 2016). Therefore, basically, membrane fouling would be relieved by protease addition in the ADMBR due to protein degradation. Dynamic membrane formed on the new understructure during 1–8 day before protease addition while it formed on the online-cleaned understructure during 10–17 day after protease addition. However, as shown in Fig. 3A, the transmembrane pressure of the former increased more quickly than that of the later, which was reflected by the slopes of the fitted line. Therefore, results demonstrate protease catalysis could indeed slightly relieve dynamic membrane fouling.

Moreover, as shown in Fig. 3B, protease catalysis was able to reduce the protein and polysaccharide contents in the sludge of dynamic membrane, which might be one of the reasons to the relieved membrane fouling. By comparing the FTIR curves, the peaks of the light transmittance were found to appear at similar wavelengths in both sludge samples before and after protease addition, which indicated there was no obvious generations of new chemicals induced by protease addition. However, the magnitudes of some peaks tended to reduce after protease addition, especially in 3415 cm^{-1}(stretching vibration of −OH in polysaccharide) (Yan et al., 2011), 1031 cm^{-1} (curved surface bending of O–H in polysaccharide) (Chen et al., 2015) and 1546 cm^{-1}(antisymmetric stretching of COO− in amino acids) (Robinson et al., 2012). Therefore, it could be qualitatively determined that protein and polysaccharide contents were reduced in the sludge of dynamic membrane by protease addition.

3.4. Stimulation of protease catalysis to microorganism hydrolase activities

Protease catalysis could simultaneously improve protease and β-GLC activities of the sludge system in ADMBR. As shown in Fig. 4, proteases activity was enhanced from about 5 to around 21 U/ml by protease addition, slightly lower than the theoretical value (μ) of 24 U/ml (obtained by model 1), which possibly resulted from the influence of some compositions in WAS, such as metal ions. However, though proteases activity in the effluent also increased after protease addition, indicating that a part of protease was lost via effluent, the proteases activity in reactor could be basically kept

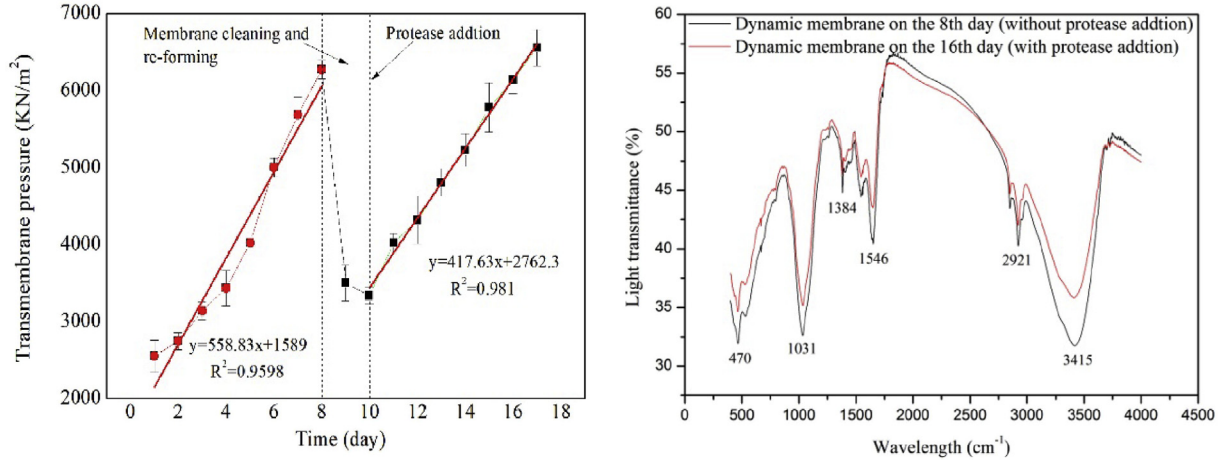

Fig. 3. Influence of protease catalysis on dynamic membrane fouling and composition.

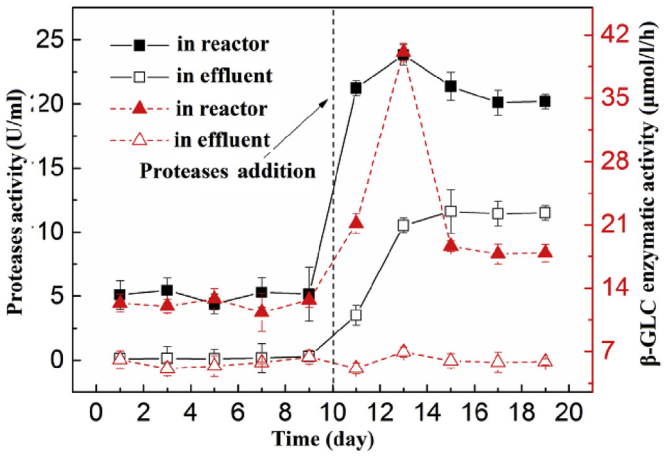

Fig. 4. Influences of protease catalysis on microorganism hydrolase activities.

stable, which thus indicated the increase of proteases activity in reactor should be contributed not only by the introduction of additional proteases, but also by the enhancement of indigenous protease activities. The improved biodegradability of those refractory proteins by protease catalysis provided abundant substrates for proteolytic bacteria and then stimulated their activities.

Furthermore, as shown in Fig. 4, it could be interestingly found that β-GLC activity was also improved by protease catalysis in the ADMBR. The β-GLC activity was only about 13 μmoL/L/h before protease addition, but it quickly raised to 41 μmol/L/h on day 13 after protease addition and finally stabilized at 20 μmol/L/h. The sharply increase of β-GLC activity should attribute to the appearance of abundant bioavailable carbohydrates after protease addition, by the process of which, a large amounts of refractory organics, such as glycoprotein, were suddenly degraded into carbohydrates, stimulating the activity of sugar-degrading microorganisms. However, due to the limited amount and rapid decrease of carbohydrates, β-GLC activity could not keep at high level and then quickly reduced after day 13. Therefore, results further confirmed that the enhanced VFAs production should directly attribute to the enhancement of microbial activity in sludge, but the externally added protease.

3.5. Influence of protease catalysis on microbial communities

3.5.1. Microbial community diversity indices

To investigate the influences of protease catalysis on microorganisms during sludge fermentation, microbial communities were analyzed in different samples, namely sludge in the reactor before (A1, on day 10) and after (A2, on day 20) protease addition, and sludge on the surface of dynamic membrane before (M1, on day 10) and after (M2, on day 20) protease addition. Firstly, as shown in Table 1, all coverage values of the four samples exceeded 99%, indicating that the results of those sequencing tests could represent the real situations of the samples. Then, it could be found that both of the OTU values of the microorganisms in the reactor and on the membrane surface decreased after protease addition, which indicated that protease catalysis could reduce microbial populations. That is, protease catalysis could not only stimulate residual organics degradation in the fermented sludge, but also partly make the cells of some living bacteria rupture. Moreover, as shown in Table 1, there are significant differences in the diversity indices, namely ACE and Simpson, between the different samples. Results indicate protease addition could slightly reduce bacterial richness and evenness in the fermentation sludge.

Table 1
Microbial community diversity indices used in this study.

Sample ID	Labels: 0.97[a]			
	OTU	ACE	Simpson	Coverage
A1	256	257.92	0.142	99.91%
M1	240	259.30	0.097	99.97%
A2	215	228.99	0.287	99.89%
M2	248	251.39	0.277	99.91%

[a] Diversity values greater than 0.97 are significant. OTU is operational taxonomic units.

3.5.2. Phylum-level taxonomic distribution

As shown in Fig. 5A, microorganisms in four samples presented similar taxonomic distribution at phylum-level, mainly including *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, RF* and *Spirochaetae,* and protease catalysis could inhibit the growth of microorganisms in *Proteobacteria* phylum and promote microorganisms in *Bacteroidetes* and *Actinobacteria* phylums. Before protease addition, microorganisms in reactor mainly distributed in the phylums of *Bacteroidetes, Firmicutes* and *Proteobacteria,* which occupied 45%, 30% and 20% of the total biomass, respectively. All of them are common microorganisms existing in traditional anaerobic fermentation reactors. After protease addition, though microorganisms in the phylums of *Bacteroidetes* and *Firmicutes* were dominant, the proportion of *Bacteroidetes* increased to 55% while *Proteobacteria* reduced to 5%.

Moreover, as shown in Fig. 5B, the agglomeration of A2 and M2 was obviously better than that of A1 and M1. Therefore, result indicated that although the microorganisms on membrane surface and in reactor were homologous, their community structure became more similar after protease addition. Furthermore, as mentioned above, microorganisms in *Bacteroidetes* and *Firmicutes* phylums were the dominant in all of the four samples, while the arrowhead indicating *Firmicutes* was close to the point of M1. Therefore, result showed microorganisms in *Firmicutes* phylum had great influence on the sludge system of membrane surface before protease addition. Finally, by comparing with the situation after protease addition, the microorganisms on membrane surface before protease addition did not present obvious higher evenness, but had more species and greater variation of community structure. It may be resulted from the abundant organics accumulated on the membrane surface, such as protein and polysaccharide, which stimulate the growth of microorganism on membrane surface. Protease addition accelerates the degradations of protein and polysaccharide on membrane surface, thus resulting in the homoplasy of microorganisms in reactor and on membrane surface.

3.5.3. Genus-level taxonomic distribution

Protease catalysis could accelerate the growth of polysaccharides hydrolyzing bacteria. As shown in Table 2, the microorganisms in *vadinBC27 wastewater-sludge group* kept dominating in all samples before and after protease additions, which was in accordance with previous reports (Liu et al., 2016a), and protease catalysis could further improve its proportion from about 36% to 52%. Microorganisms in *vadinBC27 wastewater-sludge group* was reported to be the most ruling genus detected in degrading the recalcitrant organics during the treatment of pharmaceutical wastewater (Xu et al., 2012). In this study, by protease catalysis, some non-degrading organics became difficult-degrading organics, which might promote the growth of microbes in *vadinBC27 wastewater-sludge group.* Moreover, protease catalysis could greatly promote the growth of microorganisms in the genus of *OPB54 no rank* and *Corynebacterium,* the growth rates of which might be very

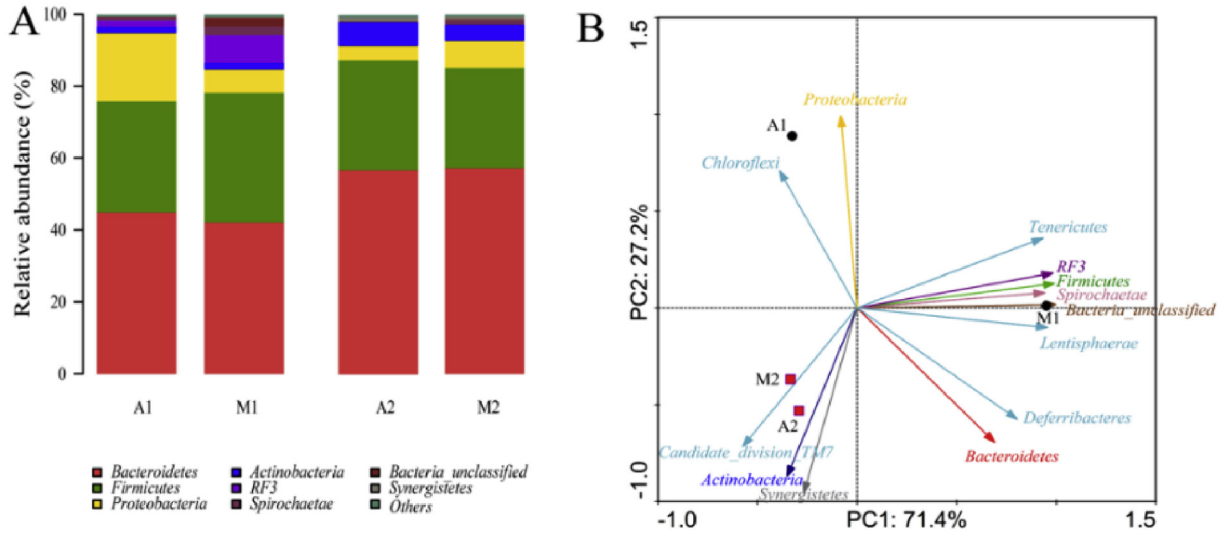

Fig. 5. Phylum distribution of the main bacteria phyla. (A) Relative abundance of the dominant bacterial phyla in the four types of fermentation sludge. (B) Principal component analysis (PCA) of phylum abundance data using Canoco 4.5.

Table 2
Proportions of microbes in the phylum, class and the genus levels.

Phylum level	Class level	Genus level	Relative abundance of 16S rRNA gene sequences (%)[a]			
			A1	M1	A2	M2
Bacteroidetes	*Bacteroidia*	*vadinBC27 wastewater-sludge group*	36	30	52	52
	Bacteroidia	*Proteiniphilum*	2	2	1	1
	Bacteroidia	*Paludibacter*	1	2	/	/
	Bacteroidia	*Petrimonas*	1	2	/	/
	SB-1	*SB-1 no rank*	1	3	/	/
Firmicutes	*OPB54*	*OPB54 no rank*	3	3	16	8
	Clostridia	*SRB2 no rank*	3	5	2	2
	Clostridia	*Anaerobranca*	2	3	2	2
	Clostridia	*Ruminococcaceae unclassified*	2	2	/	/
	Clostridia	*Clostridiisalibacter*	2	/	/	/
	Clostridia	*Clostridiales unclassified*	1	2	/	/
	Clostridia	*vadinBB60 no rank*	/	1	/	/
	Clostridia	*incertae sedis*	/	1	/	/
	Clostridia	*Proteiniborus*	/	/	1	/
	Bacilli	*Bacillus*	2	/	2	3
	Erysipelotrichia	*Erysipelotrichaceae uncultured*	/	2	/	/
Proteobacteria	*Gammaproteobacteria*	*Acinetobacter*	14	/	1	5
	Betaproteobacteria	*Alcaligenes*	2	4	/	/
Actinobacteria	*Actinobacteria*	*Corynebacterium*	1	1	6	3
Synergistetes	*Synergistia*	*Synergistaceae uncultured*	/	/	1	/
Spirochaetae	*Spirochaetes*	*LNR A2-18 no rank*	/	2	/	1
RF3	*RF3*	*RF3 no rank*	1	7	/	/

[a] Not detected.

low since they were also enriched by the help of membrane separation in ADMBR (Liu et al., 2016a). The latter mainly included alkaline anaerobic and chemical heterotrophic bacteria, some species of which could degrade glucose for lactic acid production (Lee et al., 1985). The enhanced β-glucosidase activity in ADMBR after protease addition (as shown in Fig. 4) might be relative to the increase of their biomass.

Moreover, protease catalysis could inhibit the growth of some proteolytic bacteria, causing the abundances of some genus for protein hydrolysis to reduce, such as *Proteiniphilum, SRB2 no rank* and *Acinetobacter*, and even to disappear, such as *Paludibacter, SB-1 no rank, Ruminococcaceae unclassified, Clostridiisalibacter, Clostridiales unclassified, vadinBB60 no rank, incertae sedis, Erysipelotrichaceae uncultured, Alcaligenes* and *RF3 no rank*. For example, *Proteiniphilum* could produce acetic and propionic acids by using protein-like matters as the substrate (Kobayashi et al., 2007), but could not enrich by the help of membrane retention (Liu et al., 2016a). *Acinetobacter* with optimum growth temperature of 35 °C demanded a long period of over 10 days to enrich by the help of membrane retention (Liu et al., 2016a) and could secrete proteolytic enzymes (Chen and Dong, 2005; Antunes et al., 2011). Some anaerobic bacteria in *Alcaligenes* could utilize amino acids in case the nitrate or nitrite was present (Matokwe et al., 2016; Fitamo et al., 2017). Results indicate microorganisms in most of those inhibited genus by protease addition belong to protein hydrolytic bacteria.

Finally, there were also a few of genus in which the microorganisms seemed to be resistant to protease catalysis, such as *Anaerobranca* and *Bacillus*. The former belonged to alkaline thermophilic anaerobic microorganisms and could produce acetic acid

by utilizing protein or carbohydrates (Gorlenko et al., 2004; Zhang et al., 2010). In the latter, many microorganisms could secrete several kinds of enzymes (Kobayashi et al., 2007). Both of them were not influenced by the introduction of dynamic membrane in sludge fermenter (Liu et al., 2016a).

3.5.4. Redundancy analysis

Redundancy analysis (RDA) was also adopted to investigate the influence of protease catalysis on bacterial communities in fermentation sludge. The change of protease activity, induced by protease addition, was thus used to denote the efficiency of protease catalysis. As shown in Fig. 6, the results of RDA further confirm that protease catalysis indeed influences bacterial communities in fermentation sludge. Moreover, protease catalysis makes the microorganisms on membrane surface and in the reactor tending to be analogous. The difference of microbial communities' diversities between A1 and M1 is greater than that between A2 and M2. That is, protease addition could inhibit the growths of the general microbes and indirectly accelerate the enrichments of some specific microorganisms due to the reduced competitors.

3.6. Mechanism of protease catalysis for improvement of VFAs production in ADMBR

Obtained results indicate that the mechanism of protease catalysis for further improvement of VFAs production from sludge fermentation in ADMBR could be summarized as three aspects: (1) a part of the accumulated refractory matters, such as protein, rejected by the dynamic membrane was hydrolyzed into protein fragments and small-molecule carbohydrates, which could be utilized by acidogenic bacteria mainly for VFAs production. (2) Some microorganisms would be inhibited or even broken by protease catalysis while some specific microbes, such as *OPB54 no rank* (Table 2), could survive and finally flourished by the helps of the relatively abundant substrate and less competition. (3) The total amount of the bacteria for protein hydrolysis reduced while the overall activity was positively stimulated, which might be resulted from the stronger protein hydrolysis ability of those survived protein-hydrolyzing microbes.

As shown in Fig. 7, in the conventional ADMBR system, there are abundant of refractory protein and other organics rejected by the dynamic membrane retention, and traditional anaerobic

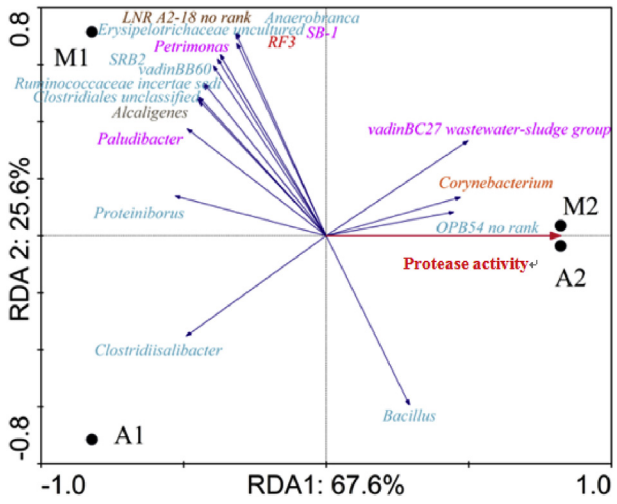

Fig. 6. Redundancy analysis of the influence of protease catalysis on bacterial communities in ADMBR.

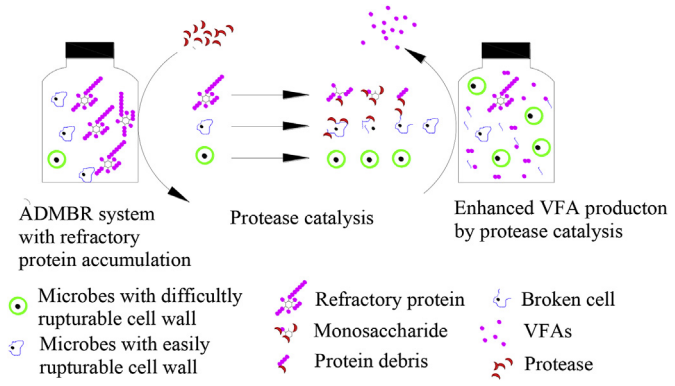

Fig. 7. Mechanism of protease catalysis in further improving VFAs production from sludge fermentation in ADMBR.

microorganisms dominates the fermentation system. Then, after the protease addition, the residual protein in fermented sludge could be partly decomposed into biodegradable matters, some microbial cells, as a kind of organics, would also be broken and partly hydrolyzed into biodegradable matters, and another kind of microorganisms, immune to protease catalysis, could quickly proliferate and continue to produce VFAs from those re-hydrolyzed organics. Eventually, protease catalysis in ADMBR improves the VFAs production and accelerates the shifts of microorganism communities.

4. Conclusion

The application of protease catalysis could ensure the stable long-term operation of ADMBR. Firstly, VFAs yield from sludge fermentation was further improved by more than 40% through the deep exploitation of refractory organics. It was interestingly found protease addition could not only improve proteases activity, but also enhance β-GLC activity of the fermentation sludge. Secondly, membrane fouling was slightly relieved possibly due to the degradations of protein and polysaccharide in the sludge of dynamic membrane. Finally, the function of fermentative microbial system could be maintained, though bacterial richness and evenness reduced. As a kind of survival pressure, protease catalysis could accelerate the growth of polysaccharides-hydrolyzing bacteria and inhibit some proteolytic bacteria, but the survived bacteria from the latter possibly possessed high hydrolysis activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jenvman.2018.03.103.

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