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Original Article

Enhanced volatile fatty acids production from Napier grass *(Pennisetum purpureum)* using micro-aerated anaerobic culture♣

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Abstract

The laboratory-scale horizontal acidogenic bioreactor was conducted in semi-anaerobic and strict anaerobic conditions using Napier grass (*Pennisetum purpureum*) as the sole substrate to determine the effects of micro-aeration on organic acids yield. During semi-anaerobic condition, total volatile fatty acids (VFAs) yield was significantly higher (α=0.05) i.e. 3,524±191, and 2,831±89 mg/L for the micro-aerated, and anaerobic conditions, respectively. The biomethane production was effectively suppressed during micro-aeration as a result of methanogenesis inhibition by the controlled oxygenation. Improved hydrolysis by facultative organisms was evidenced by the elevated soluble chemical oxygen demand concentration which was readily available for organic acid production. Hemicellulose in the biomass was efficiently degraded at 10.0% during micro-aerated period, while other constituents i.e. cellulose and lignin remained in the digestate. Potential use of these co-products of VFA-rich liquor and pretreated solid digestate for higher biofuels or valuable biochemical compounds was discussed in the context of the bio-circular-green economy model.

Keywords: micro-aeration, lignocellulosic biomass, volatile fatty acids, digestate, anaerobic digestion

1. Introduction

Anaerobic biorefinery is a relatively new concept in which anaerobic digestion serves as a center piece to generate high-value products ranging from biofuels (hydrogen, ethanol, and butanol) to many bio-based products (i.e. bioplastic and biochemicals) instead of a conventional methane gas. Volatile fatty acids (VFAs), organic acids with carbon 2 to 5 atoms, can also be used as an excellent carbon source for denitrifying bacteria (replacing methanol) for biological nutrient removal (Elefsiniotis & Wareham, 2007) or lipid-accumulating microalgae (e.g. *Chlorella protothecoides* and *Cryptococcus albidus*) that contains high amounts of omega-3 fatty acids and exopolysaccharides (Chalima *et al.*, 2017). The main idea of this notion is to optimize the profit of an AD system by producing high-value bio-based products while serving the energy needs by producing biofuels in subsequent stages. In addition, eliminating methanogenesis stage favors the production of VFAs as well as shortens substrate residence time in reactors. Capital and operating costs can be cut down leading to increased economic feasibility of the system. A variety of substrates including agricultural residues (Zacharof & Lovitt, 2013) and vegetable wastes (Li *et al.*, 2017) were successfully used to produce VFAs by various strategies to inhibit VFAs conversion. The shift in the generation as well as

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composition of VFA species was also found to respond to operating parameters, i.e. pH, organic loading rate (OLR) and hydraulic retention time (HRT), in connection with the different types of substrates digested (Bengtsson, Hallquist, Werker, & Welander, 2008; Chen, Jiang, Yuan, Zhou, & Gu, 2007). This is worth further investigations as it has implications on the values of the total end products. Since operating digester at shorter HRT or high OLR favors acidogens to shift the final product to VFAs instead of methane. Recently, aeration to the anaerobic digester has been suggested as a new way to stimulate the growth of facultative microorganisms which could then enhance hydrolysis and acidogenesis. Thus, the concept of micro-aeration to AD was applied to increase the excretion of extracellular enzymes to enhance hydrolysis-acidogenesis reaction chain. Several researchers reported the enhanced hydrolysis of many organic materials including lignocellulosic biomass) and sewage sludge (Johansen & Bakke, 2006; Sawatdeenarunat, Sung, & Khanal, 2017) by micro-aeration. Zhu, Lü, Hao, He, and Shao (2009) revealed that the acidogenesis efficiency correlated to the intensity and cycle of aeration during anaerobic digestion of fresh vegetable and flower wastes. However, excessive air dosage caused negative impacts on VFAs yield. Extended retention of the substrate with suitable aeration could be the key for continued VFAs production without acclimation of methanogens to stresses such as low pH or OLR since methanogens are strict anaerobes. With ample information on batch studies, long-term stable operation with such micro-aeration in continuous anaerobic digestion of lignocellulosic biomass using horizontal bioreactor to prevent scum formation is still lacked.

Therefore, the main purpose of this study is to evaluate the effect of micro-aeration on the VFAs production from Napier grass using the continuous operation of horizontal bioreactor. Changes in biogas composition and yields were monitored in relation to the process outputs, i.e. biomass composition and organic acids generation and composition. Finally, the benefits from acid production were assessed as a potential gain from our proposed micro-aeration strategy.

2. Materials and Methods

2.1 Substrate and inoculum

The 3-month-old Napier grass was harvested from the University of Hawai'i Waimanalo Research Station (Waimanalo, HI, USA). The harvested biomass was processed to the final size of 6 mm and moisture content less than 10%. Finally, the grass was kept in a vacuum bag. The total solids (TS), volatile solids (VS), and fiber composition of the biomass, i.e. neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), were analyzed. The characteristics of Napier grass used in this study were 91.0±1%, 83.0±1%, 39.5±0.9%TS, 25.3±1.6%TS, and 10.2±0.3%TS for TS, VS, cellulose, hemicellulose, and lignin, respectively. The inoculum was anaerobically digested cattle manure (ADCM) which was prepared in the laboratory using the 20-L anaerobic reactor fed with cattle manure and operated at mesophilic conditions (33±2˚C) for over a year. The initial seed sludge concentration of the reactors was 25,000 mg/L as volatile solids.

2.2 Reactor configuration

Two acrylic horizontal bioreactors with the radius of 0.15 m, and the length of 0.30 m resulting in the total volume of approximately 5 liters were used in this study as shown in Figure 1. Each reactor has a working volume of 2.7 L. The horizontally mechanical mixers were installed to mix the reactor contents, move them to the outlet port, break down an accumulated scum, and degas the liquid. The two reactors were operated as duplicate at mesophilic condition (33±2 °C) where the average values were used in this paper. A silicone rubber heating blanket (BriskHeat OH, USA) installed to maintain the reactor temperature. The thermocouple type T (Omega Engineering, Inc., Connecticut, USA) and data locker (Dataq Instruments, Inc., Ohio, USA) were used to monitor and control the reactor temperature. The reactors were operated in semi-continuous mode. The mixing speed was fixed at 90 rpm and turned on for 5 minutes in a 30-minute cycle. There are four air injection ports installed at the bottom along the length of reactor.

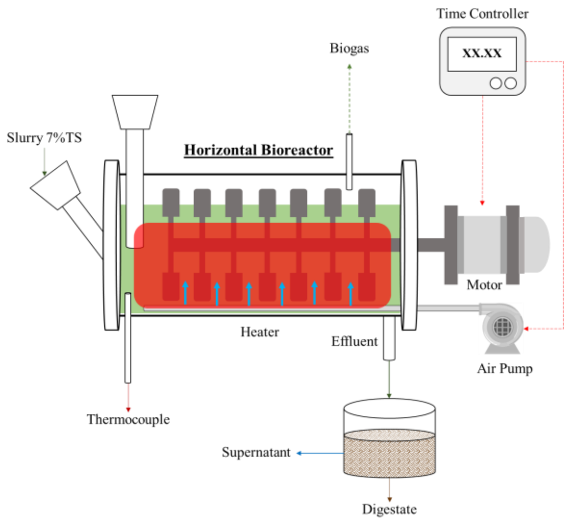

Figure 1. Horizontal acid bioreactor used in the experiment

2.3 Reactor operation

The bioreactors were started up at an initial OLR of 4 kgVS/m^3.d for a month. The feedstock was prepared according to section 2.1. The slurry of feed prepared at 7% was fed thru the inlet port at the same time once a day. During operation, the reactor contents were withdrawn at the outlet port and analyzed as detailed in section 2.4. During the reactor operating period, the operating OLR was stepwise increased at an interval of 1 kgVS/m^3.d until reactor failure (high accumulation of solids) was observed at OLR 7 kgVS/m^3.d. OLR was then suddenly reduced to 6 kgVS/m^3.d (HRT of 14 days) and continued until stable operation was reached. Subsequently, the micro-aeration was applied thru fine diffusers for 1 min every 6 hours using an air pump (Super pond, WA, USA) at a constant flow rate of 450 mL/min. The appropriate air flow rate was fixed by the pump and the authors had run a preliminary study at a few aeration frequencies. At higher frequency (i.e. every 2 hours), the VFA production was not enhanced while the methane in the reactor's headspace was diluted. At lower frequency, no clear effect on the VFA production was detected. Thus, the micro-aeration frequency at 6 hours was selected for our experiment. The reactors were operated under micro-aeration mode until stable which lasted 50 days. Effluents were collected for analyses of both liquid and solid portions for comparison with those from anaerobic condition.

2.4 Analytical methods

A bench top pH meter (Accumet AB15, Fisher, Fairlawn, USA) was used to analyze pH of the rector contents. Soluble chemical oxygen demand (SCOD**),** TS, and VS were determined using the Standard Methods (APHA, 2005). NDF, ADF and ADL of the biomass before and after AD were analyzed as described by (Madrid, 2004). Cellulose and hemicellulose were calculated using the following equations: Hemicellulose = NDF – ADF and Cellulose = ADF – ADL. A milli-gas-counter (Ritter US LLC. NY, USA) and gas chromatography with thermal conductivity detector (GC-TCD) (GC2014, Shimadzu, Japan) equipped with a packed column (80/100 Hayesep D column, 2 m length × 3.2 mm outer diameter × 2.1 mm inner diameter, Supelco, USA) were used to determine the produced biogas, and biogas composition, respectively. The individual VFAs were analyzed using GC with flame ionization detector (GC-FID) (GC2014, Shimadzu, Japan) using a capillary column (ZB-Wax Plus column 30 m length × 0.25 mm inner diameter × 0.25 µm film thickness, Phenomenex, USA).

2.5 Statistical analysis

The analysis of variance (ANOVA) and Tukey's test for post-hoc analysis was used to examine statistically significance with a threshold value $\alpha=0.05$ using JMP statistical software (JMP Pro 12.0.1, SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1 VFAs yield during AD of Napier grass

The OLR was gradually increased by 1 kgVS/m^3.d from 4 to 7 kgVS/m^3.d within approximately 100 days. The signs of system imbalance started to occur at OLR 7 kgVS/m^3.d, i.e. the ratio of volatile fatty acids to alkalinity (VFA/ALK) over 1.0 and lowering effluent pH value. It is noted that the maximum VFA/ALK of 0.8 is recommended for stable anaerobic digester of various substrates (Khanal, 2008). During such period, $NaHCO_3$ was added to correct the buffering capacity and pH of these duplicate reactors. After a few attempts, it was concluded that system failure at OLR 7 kgVS/m^3.d was permanent. The operating OLR returned to 6 kgVS/m^3.d and the bioreactors were operated at stable condition for more than a month before the data collection and analysis were performed. Ambient air was injected to the reactor content at day 57 into the operation at OLR 6 kgVS/m^3.d. After micro-aeraion (1 minute every 6 hours), the interesting changes in VFAs concentrations appeared together with the fluctuating pH, as shown in Figure 2.

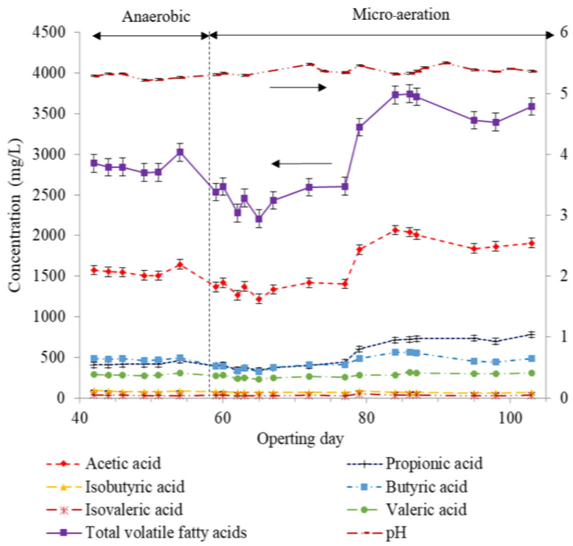

Figure 2. pH and VFAs composition in the effluent before and after micro-aeration at the operating OLR of 6 kgVS/m^3.d

At stable operating condition (OLR of 6 kgVS/m^3.d), pH of the effluents was at virtually the same level at 5.27±0.04 and 5.37±0.06 for anaerobic and micro-aerated conditions, respectively. These values were a little lower than the recommend range for acid bioreactor (i.e. 5.5-6.5) (Agler, Wrenn, Zinder, & Angenent, 2011) where methanogenic growth is largely inhibited . The total VFA concentration (TVFA) was between 2,800 and 3,030 mg/L during day 42-57 (anaerobic period). However, after micro-aeration at day 57, TVFA significantly decreased to between 2,500 and 2,600 mg/L during days 59 to 77. This phenomenon clearly indicated an acclimation of the microorganisms toward micro-aerated condition. The chain of reactions was disrupted, which caused harsher effect to the strict anaerobes of methanogens and trickled up to the facultative hydrolytic and acidogenic organisms in front end reactions. It took 18 days for the facultative organisms to adapt. After which, there was a big leap of VFAs, indicating an enhanced growth of cellulolytic organisms. It was clear that such increased hydrolysis was coupled with a suppressed methanogenic activity. This phenomenon might be from the acclimation of the microorganism toward micro-aerated condition (Yu *et al.*, 2020). Continued operation of micro-aeration scheme approximately 20 days has caused the significant shift in anaerobic pathways in the horizontal bioreactors. The VFAs concentration sharply increased after day 77 by 43% from 2,600 to 3,730 mg/L within only two days. At stable condition, it was obvious that TVFA concentration of the micro-aerated operation was significantly higher than that of anaerobic condition (i.e. 3,524±191 vs. 2,831±89 mg/L). It was due to the biological conversion of organics by oxidative pathway of facultative organisms in the culture. Such phenomenon was supported by our observation in the drop of methane and the rise of carbon dioxide content in the biogas. Methane content in the produced biogas dropped from 16.8±2.2% in anaerobic condition to 6.6±1.7% in micro-aerated condition. During this period, methanogenic archaea was inhibited by the presence of molecular oxygen in the environment in addition to the low pH environment they reside.

Our results agreed with the previous batch studies but at a lesser degree of VFA increase. Studies of micro-aeration using lignocellulosic materials i.e. grass silage as substrates reported 4-fold in VFAs production after micro-aeration (Jagadabhi, Kaparaju, & Rintala, 2010). It could be due to the difference between continuous versus batch operation where maximum increase was compromised by the constraints of reactor operations such as hydraulic retention time and organic loading. Too long of the oxygenation to the digester although at batch optimal oxygen dosage of 0.1 volume air per volume slurry per minute caused a severe drop in the acetate produced after 60 hours of digestion time (Obeta Ugwuanyi, Harvey, & NcNeil, 2005). This gap of hydrolysis-acidification improvement can be closed once more studies embarking the optimized micro-aerated acidogenic operation is carried out in greater details. There is still a wide opportunity for future studies on this micro-aeration acid reactor.

Analysis of the composition of VFA species produced during the anaerobic and micro-aerated condition showed that acetic acid still dominated for more than 50% of the total VFAs in both anaerobic and micro-aerated conditions. Other VFAs (i.e. propionic, butyric, and valeric acids) observed during these operations were increased in accordance with the acetic acid, but the concentrations of minor VFAs species i.e. iso-butyric, iso-valeric, and valeric acid were not significantly different. This was consistent across other operations of digester configuration with grass biomass feedstock as well, such as the batch-type leach-bed reactor where acetic acid constituted for 40% of total VFAs in leachate (Jagadabhi *et al.*, 2010). In such micro-aerated leach-bed operation, there did not appear to be acetogenic inhibition since acetate was still produced at high level. Although inhibition of specific VFA species to methanogens was widely reported by Zhao *et al.* (2020), to our knowledge, there is no specific report on the VFA species on acidogenic activity other than product inhibition (Sui *et al.*, 2018). This study is worth further investigations.

3.2 Soluble chemical oxygen demand (SCOD) production

Typically, SCOD could indicate the soluble products from hydrolysis stage of AD. The produced SCOD and VFAs during micro-aerated condition were 10 and 24% higher than those of anaerobic condition, respectively. The SCOD and TVFA concentrations in the reactor contents were illustrated in Table 1. According to the increase of SCOD and VFA, the ratio of VFAs and SCOD of micro-aerated condition also rose 11% higher than that of strict anaerobic condition. VFAs/SCOD ratio is indirectly used to determine the efficiency of acidogenesis stage of AD systems. This parameter could be used to represent an efficiency of acidogens to convert the hydrolyzed products (i.e. alcohol, sugars, amino acid, and fatty acids among others) in the reactor contents to VFAs.

Enhanced hydrolysis during aerated AD of many organic substrates and bioreactor configurations was reported by many researchers. The positive effect on hydrolysis of protein and carbohydrate during micro-aerated AD of primary sludge was presented by Johansen and Bakke (2006). More than 50% increase in COD during hydrolysis was detected compared to normal anaerobic condition. Under micro-aeration in thermophilic digester operation, an increase in hydrolytic enzymes such as cellulase and protease production by the AD microbial culture was found to be a factor to enhance acidogenesis (Jagadabhi, Kaparaju, & Rintala, 2010). However, the air dosage plays a key role in the productivity of hydrolysis reaction. An inappropriate aeration dosage (i.e. insufficient dosage) could also reduce the hydrolysis efficiency compared to that of unaerated condition, as reported by Zhu *et al.* (2009). The facultative microorganisms would be more active during micro-oxygenation as a result of enhanced extracellular hydrolytic enzymes production (Bengtsson *et al.*, 2008). Some microorganisms are augmented during micro-areared condition depending on the flora existed in the substrate; for example, higher microbes in *Firmicutes* phylum were the dominent group of food waste and brown water digestion with some oxygenation (Zhu *et al.*, 2009). However, too high of the oxygen would lead to the revival of aerobic organisms as well. The balance of oxygenation rate to different kinds of substrates and reactor configurations is of great interest for further development.

3.3 Biogas composition

Multiple reports indicated that aeration inhibited the strict anaerobic microorganisms (i.e. methanogens) (Sui *et al.*, 2018) and allowed the alternate production of other valuable products (e.g. hydrogen and VFAs) particularly during acidogenesis stage of AD (Sołowski, Konkol, & Cenian, 2020). Our results show that methane yield under the anaerobic condition was significantly lower (α=0.05) than that of the micro-aerated condition. However, no significant difference of CO_2 yield was observed among the two operating conditions. This phenomenon was explained by the inability of methanogenic species to synthesize *superoxide dismutase* which is the enzyme responsible for mitigating oxygen ion and radical toxicity (Botheju & Bakke, 2011). Many methanogen cells such as those of *Methanococcus voltae* and *Methanococcus vannielii* could be severely

Table 1. Soluble chemical oxygen demand (SCOD) and volatile fatty acids (VFAs) production during stable operations before and after micro-aeration

Condition	SCOD (mg/L)	VFAs (mg/L)	VFAs/SCOD	CH_4 yield (NmL/g VS_{added})	CO_2 yield (NmL/g VS_{added})
Anaerobic	8017±1039	2831±89	0.36±0.04	13.7±1.8*	21.7±3.0
Micro-aeration	8839±482*	3524±191*	0.40±0.03*	8.5±1.8	20.8±2.9

Note: Values are presented in average ± SD (n =10); * significantly different at α=0.05

damaged when exposed to oxygen (Kiener & Leisinger, 1983). The average CH_4 and CO_2 yields during the operations are illustrated in Table 1.Analogues to this study, Botheju and Bakke (2011) revealed that in the batch experiment, the aerated inoculum could result in three times longer lag phase of methanogenation compared with that of the unaerated inoculum. The relation between the methane production and VFAs yield versus oxygenation was critical in the scheme of organic acid producing reactor as the substrate type and loading rate to the reactor must be balanced with the oxygenation for targeted optical operation (Eryildiz, Lukitawesa, & Taherzadeh, 2020). The level of methanogenic inhibition and hydrolytic-acidogenic augmentation will play an important role in valuation of the products from the process. In addition, it is noted that CH_4 content in the produced biogas was low (in a range of 15-25%) as a synergic result of air dilution and methanogenic inhibition. For CO_2 generation, by introducing the appropriate oxygen dosage, rather than converting organic matter via aerobic respiration to produce CO_2, the facultative microorganisms selectively kept metabolic pathway via fermentation to produce VFAs. However, oxygen overdosing posed a threat of increased CO_2 production, which has lowest value, as the metabolic function of facultative organisms had shifted to aerobic respiration (Botheju & Bakke, 2011). Besides, the more CO_2 production means the loss of carbon source for synthesizing organic acid molecules and waste of energy for over oxygenation. Thus, the appropriate reactor operation must preserve VFAs as the main product from the AD of Napier grass while minimizing all other competing products at the same time. Further studies on such issues are under investigations in the valuation improvement of biocircular research.

3.4 Fiber composition

Cellulose, hemicellulose (structural carbohydrates) and acid detergent lignin (ADL) of Napier grass before and after AD from both micro-aerated and anaerobic conditions are presented in Figure 3. The changing in cellulose, hemicellulose, and ADL would dictate the appropriate conversion and utilization of the digestate. Hemicellulose contents of the digestates from anaerobic and micro-aerated conditions were 26±1 and 27±1%TS, respectively. These values from both conditions were significantly lower than that of raw Napier grass (i.e. 30±0%TS). The hemicellulose removal in anaerobic operation was found to be 13.3% compared to 10.0% during the micro-aerated one. Reversely, cellulose and lignin still persisted in the digestate with negligible degradation. These constituents could be further utilized to produce solid biofuel via thermochemical processes such as hydrothermal carbonization (Funke & Ziegler, 2010) and torrefaction (Rentizelas & Li, 2016), or other biobased products (Sawatdeenarunat *et al.*, 2016). Hemicellulose was leached off the biomass well even in mild acidic condition as similarly observed during anaerobic fermentation of palm fiber (Saritpongteeraka, Chaiprapat, Boonsawang, & Sung, 2015) and Napier grass (Sawatdeenarunat *et al.*, 2017). Operating in the acidic condition functioned as a mild acid pretreatment to increase the accessibility of the hydrolytic enzymes by weakening the recalcitrant structure of lignocellulosic biomass. Typical compositions of a plant cell wall are mainly lignocellulosic materials namely cellulose, hemicellulose, and lignin. However, 5-10% of a plant cell wall could consist of other nonstructural components such as protein and lipid (Zeng *et al.*, 2017). Thus, the total percentages of lignocellulosic compositions in Figure 3 were not equal to 100%.

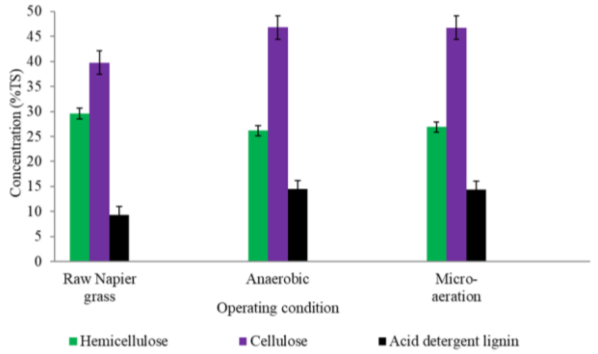

Figure 3. Fiber composition of Napier grass biomass during anaerobic and micro-aerated operations.

3.5 Value chain perspective

To optimize the benefit of the system, the downstream purification processes are required to obtain only VFAs as a product. Many physical and chemical techniques have been reported for purifying the anaerobic mixtures (Zacharof & Lovitt, 2013). The individual VFAs could be used as initial substrates to produce many specific high-value biobased products. For example, acetic acid, the major produced acid in this study, could be used to produce a food additive and favoring agents in some food industries. Propionic acid could also be potentially served as food and feed preservative compounds and used to produce vitamin E (Yang, Wang, Lin, & Yang, 2018). The well-known biodegradable plastics; polyhydroxyalkanoates (PHA), and polyhydroxybutyrate (PHB) could specifically be produced from acetic acid and butyric acid. The market size and price of the three main VFAs are presented in Table 2. It should be noted that the dominant individual VFAs species during AD of Napier grass in this study are acetic acid and propionic acid. By their large market size globally and production potential from the abundant biomass, the technical appropriateness of the extraction technologies and their associated costs will only be a limit and plays an important role in this bio-based business. In addition, the values of the produced individual acids per ton of dry Napier grass obtained in this study are showed in Table 2. Acetic acid showed the highest worth of 310 USD/dry metric ton of grass following by butyric acid and propionic acid. For the supply side of the feedstock, other biomasses (i.e. cheap cultivated crops, agricultural residues, or wastes) can be used as starting material for the acid producing reactor and more research to convert these different organic masses to high value compounds are underway.

4. Conclusions

The VFAs yields in the horizontal bioreactors operation using Napier grass feedstock were enhanced by

Table 2. Volatile fatty acids market size and price

Volatile fatty acid	Global market size (metric ton/year)	Unit price* (USD/metric ton)	Market value (Million USD/year)	Estimated value in this study** (USD/ dry metric ton of grass)
Acetic acid	3,500,000	400-800	1400-2800	310
Propionic	180,000	1500-1650	270-297	157
Butyric	30,000	2000-2500	60-75	174

micro-aeration. A significant increase of VFAs concentrations at the operating OLR of 6 kgVS/m^3.d was observed. The strict anaerobe methanogens were hindered by micro-aeration while augmenting hydrolytic and acidogenic organisms. The VFAs produced from micro-aerated AD system are the primary substrate to synthesize the high-value bio-based products in the bio-circular-green technology platform to enhance the economic viability of anaerobic digestion systems.

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