MICROBIAL CONVERSION OF SEWAGE SLUDGE FOR ADVANCED FUEL PRODUCTION

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ABSTRACT

Anaerobic digestion is widely used as a waste management practice to treat organic wastes such as agriculture or human wastes and generate renewable energy – biogas. Typical anaerobic digestion includes four consequent steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis to complete the degradation of organic compounds and produce methane. In this study, anaerobic digestion was integrated with fungal and engineered bacterial cultivation to accumulate lipid or fatty acids for biodiesel production. The anaerobic digestion was first modified by inhibiting methangoens to enable bacterial community dominate the anaerobic culture and produce volatile organic acids. 4.3 $g \cdot L^{-1}$ acetate were accumulated from the modified anaerobic digestion. A fungus (Mortierella isabellina) and an engineered bacterium (E. coli) were then cultured on the acetate from the modified anaerobic digestion to accumulate lipids and fatty acids (resources of biodiesel fuel). The engineered E. coli had a fatty acid conversion of 16% of theoretical value much higher than 7.5% from the fungus of M. isabellina. This study demonstrated a potential path that combines engineered bacterium with modified anaerobic digestion to biologically convert organic wastes into advanced fuels.

Key words: ACETATE, FATTY ACIDS, LIPIDS, METHANOGENS, *MORTIERELLA ISABELLINA*, *E. COLI*

INTRODUCTION

Sewage sludge is a complex mixture that contains organic, inorganic, and biological pollutants from municipal wastewaters. A National Research Council (NRC) report also estimated that approximately 5.6 million dry tons of sewage sludge is generated annually from wastewater treatment operations in the U.S. alone (www.epa.gov). Due to the concern of public health, the sewage must be treated to eliminate human pathogens before its land application and public distribution. On the other hand, sewage sludge is rich in organic compounds such as carbohydrates and proteins. It has potential to be used by various biological processes to produce value-added products.

Anaerobic digestion is such a process to efficiently utilize the sludge to produce bioenergy and fertilizer (Chen et al. 2008). Anaerobic digestion includes several biological steps: microbial hydrolysis of organic polymers (proteins and carbohydrates) into monomers (sugars, amino acids); acidogenesis and acetogenesis to convert sugars and amino acids into acetic acid and other organic acids; and methanogesis to convert organic acids to methane and carbon dioxide. Methane as main product of anaerobic digestion of the sewage sludge can be used for electricity generation. However, relatively low electricity buy-back rates (the primary revenue from

methane of AD) and relatively high capital costs for a large AD system (electricity generation for the grid) challenge the economic feasibility of AD technology for various scale operations, particularly medium and small sewage sludge operations. Thus, in order to make AD more attractive and suitable for a wide range of applications, an alternative utilization of AD wastes needs to be developed for generation of more valuable products. This study focused on combining a modified AD with fungal and bacterial fermentation to accumulate lipid or free fatty acids for biodiesel production. Biodiesel is an advanced biofuel, whose main components are fatty acid esters that is typically synthesized by chemically reacting lipids or fatty acids with an alcohol.

It has been reported that anaerobic digestion process under unfavorable digestion conditions such as low pH and existence of inhibitors was capable of degrading organic matters mainly into acetic acid and other carboxylic acids instead of methane gas (Rughoonundun et al. 2010). Acetic acid as an organic acid can be used as a carbon source to support a variety of microbes for fuel/chemical production. A fungus, *Mucor circinelloides,* can accumulate linolenic acid from acetate as the sole carbon source (Monique Immelman 1997). Christophe et al. have reported that an oleaginous yeast, *Cryptococcus curvatus,* was able to sequentially utilize glucose and acetic acid to accumulate lipid (Christophe et al. 2012). Lee et al. co-cultured two bacteria of *Clostridium butyricum* and *Rhodobacter sphaeroides* on acetic acid to produce biohydrogen (Lee et al. 2012). In this study, a lipid accumulation fungus, *Mortierella isabellina,* and a bacterium, *Escherichia coli* (wild type and engineered one) were selected by this study to utilize acetate from AD to accumulate lipids or fatty acids for biodiesel production. Correspondingly, a stepwise strategy was designed to fulfill the study (Fig. 1): 1. Modify anaerobic digestion to convert sewage sludge to acetate; 2. Apply *M. isabelina*, wild-type *E.coli,* and engineered *E. coli* on acetate to produce fatty acids for biodiesel synthesis.

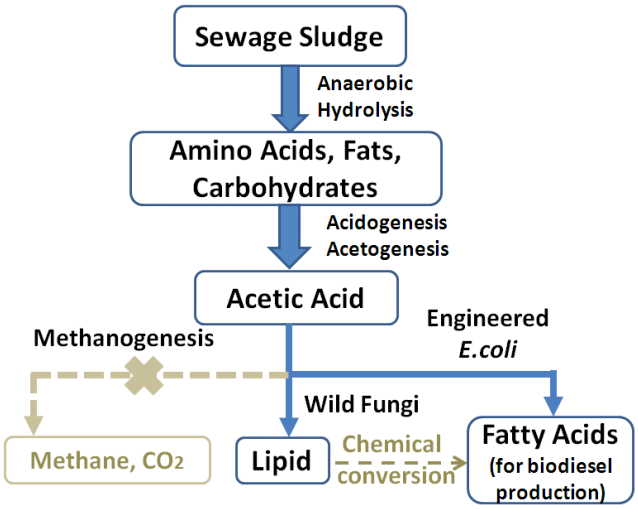

Figure. 1. Fatty acid production from anaerobic digestion acetate

METHODS AND MATERIALS

Anaerobic treatment of sewage sludge

The effluent from aeration pond was obtained from East Lansing Waste Water Treatment Plant (East Lansing, MI, USA). The effluent was centrifuged at 3273×g for 20 min to separate sludge from the effluent. The sludge was then pretreated at 100°C for 1 hr (Rughoonundun et al. 2010). A total solid of 5% of the sludge was used for acetic acid production. The anaerobic digestion was carried out using 500 ml anaerobic bottles with 400 ml pretreated sludge medium. An anaerobic seed from Michigan State University pilot anaerobic digester was added into the culture at a ratio of 12.5% (v/v) at the beginning of the culture. Two treatments including chemical inhibition (using idoform to inhibit methanogens and pH around 7.0) and pH adjustment (pH adjusted to 5.0) were carried out. Idoform solution was prepared to use pure ethanol to dilute idoform and make 20 g/L of idoform solution. 0.4 mL/L of idoform solution was added into the culture every 48 hours, while pH was

controlled using 30% (w/w) NaOH for the cultures with idoform inhabitation and 10% (v/v) hydrochloric acid for the cultures with pH adjustment (Rughoonundun et al. 2012). Feeding and sampling during the anaerobic digestion were conducted under anaerobic environment created by Simplicity 888 Automatic Atmosphere Chamber TM purchased from PLAS & LABS, Lansing, MI, USA.

Microbial fermentation for biodiesel fuel production

Mortierella isabellina ATCC 42613 was obtained from the American Type Culture Collection (Manassas, VA). The culture conditions were previously reported with slight modification (Ruan et al. 2012). 1g·L^{-1} NH_4Cl Instead of yeast extract was used as the nitrogen source. *M. isabellina* ATCC 42613 was cultured in nitrogen limited medium at three initial acetate concentrations (2.34 g/L, 4.8 g/L, 7.11g/L).

An engineered *E. coli* strain was constructed to improve the conversion of acetic acid for fatty acid production, An acs gene (acetyl CoA syntheatase, for acetic acid assimilation) and a tesA gene (acyl-ACP thioesterase, for fatty acid production) from *E. coli* genome were cloned into pUC19K (a derivative of pUC19) and introduced into *E. coli* host (Unpublished data). The mutant *E. coli* with acs and tesA genes grew on a M9 medium (containing 33.9 g/L disodium phosphate, 15.0 g/L monopotassium phosphate, 2.5 g/L sodium chloride, 5.0 g/L ammonium chloride, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 50 mM sodium acetate, 25 µg/ml kanamycin, and 0.5% YE) for ~16 hours (OD_{600}= ~3, most acetate was used for biomass production) at 37 °C to prepare the E. coli seed. Then, 1 volume of HAc-rich AD effluent (centrifuged twice at 7916 x g for 15 min at 4°C after anaerobic digestion, then autoclaved) was mixed with 3 volumes of the *E.coli* seed, and 0.2 mM IPTG was added to induce fatty acid production. The mixed culture was kept at 30°C for 24 hours before harvesting.

Analytical methods

Acetic acid concentration was quickly detected following instructions of Megazyme Acetic Acid Kit assay procedure (www.megazyme.com). HPLC with Aminex HPX-87H column (Bio-Rad Lab, Hercules, CA, USA), 65^{o}C, 0.6 ml/min, 25 min, RID, was also used to analyze acetate and other organic compounds (Ruan et al. 2012). Cell biomass was collected by filtration and washed twice with distilled water. Cell mass was determined by drying under 105±1°C overnight to obtain a constant weight. Dried cells were then ground in a mortar and used for lipid extraction according to Bligh and Dyer method (Bligh and Dyer 1959). Total lipid was determined gravimetrically. Fatty acids were measured through a modified method (Voelker and Davies 1994; Aldai et al. 2006; Lu et al. 2008; Steen et al. 2010). A GC (Hewlett Packard model 7890A, Agilent Technologies, equipped with a DB5-MS column, J&W Scientific) with a mass spectrometer (5975C, Agilent Technologies) was used to detect the fatty acid, and the carrier gas was helium.

RESULTS AND DISCUSSION

Optimization of Anaerobic Digestion for acetic acid production from sewage sludge

During anaerobic digestion, acetic acid and other organic acids as intermediates were produced by bacteria that are functioned in the processes of hydrolysis, acidogenesis, and acetogenesis (Yue et al. 2010). In regular digestion processes, these organic acids are quickly metabolized by methanogens to produce methane and carbon dioxide (Gavala et al. 2003). It has been reported that pH is one of the most important factors to influence the AD process through changing microbial communities. Low pH has significantly negative impact on methanogens and less impacts on bacteria, which consequently leads the anaerobic digestion to accumulating acetic acid and other organic acids etc. (R.J. Zoetemeyer 1982). Therefore, reducing pH of the digestion can be a simple approach to modify the anaerobic digestion to accumulate acetic acid. In addition, it has also been studied that using methanogen inhibitors such idoform is another effective way to adjust the digestion to accumulate organic acids (Aiello-Mazzarri et al. 2006; Rughoonundun et al. 2010; Rughoonundun et al. 2012). Thus, a comparison between two different AD control strategies was fulfilled to produce acetic acid from sewage sludge.

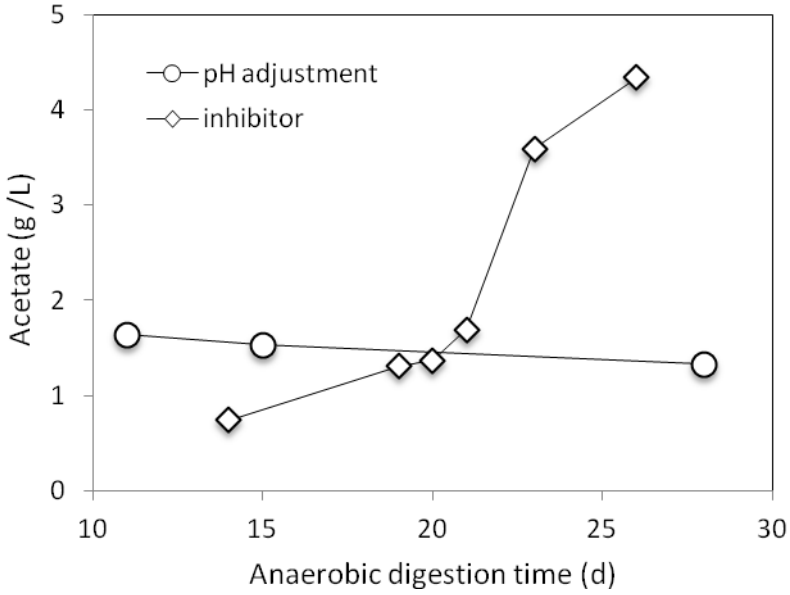

Figure 2. Acetate accumulation between pH control strategy and Inhibitor control strategy

The preliminary digestion under different pH values presented that the cultures under pH lower than 6 significantly reduced methane production (data not shown). Correspondingly, a pH of 5 was selected to evaluate the efficiency of acetic acid production using the pH control strategy. In comparison, the chemical inhibitor was applied to the digestion to improve acetic acid production from the sewage sludge at a neutral pH condition. As shown in Fig. 2, both digestions showed acetic acid accumulation. The digestion with pH control accumulated 1.64 g/L acetic acid in 11 days of the culture, and further increasing culture time did not contribute to the acetic acid accumulation. While, inhibitor control strategy demonstrated a slow start-up that the digestion only produced 0.7 g/L of acetic acid in the first 14 days of the culture. A fast growth started after 20 days. The concentration of acetic acid reached the highest one of 4.34 g/L that is approximately three times higher than the acetic acid concentration from the digestion with pH control.

Fungal lipid accumulation from acetic acid as sole carbon source

A fungus (*Mortierella isabellina*) and an engineered bacterium (*E. coli*) were then cultured on pure acetate and the acetate from the modified anaerobic digestion to compare lipid accumulation.

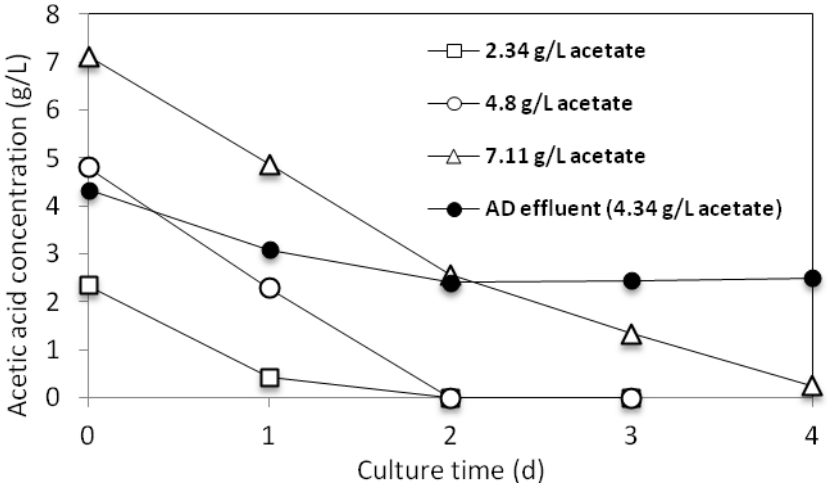

Figure 3. *M. isabellina* culture on pure acetate and acetate from AD effluent

As presented in Fig.3, *M. isabellina* can steadily consume acetate from either AD or pure acetate solution with different concentrations. At low acetate concentrations of 2.34 g/L and 4.8 g/L, acetate was consumed up within 2 days, while it took four days to consume acetate at high acetate concentration of 7.11 g/L. Even though the culture at the high acetate concentration of 7.11 g/L needed 2 more days to uptake the acetate than the acetate concentration of 5 g/L, the consumption rates between two cultures on 4.8 g/L and 7.11 g/L acetate were same in the first two days culture, and both were much higher than the culture on 2.34 g/L acetate. Meanwhile, the lipid contents in fungal biomass demonstrated that higher acetate concentration of 7.11 g/L had the highest lipid content of 0.23 g/L compared to 0.05 g/L and 0.15 g/L from acetate concentrations of 2.34 g/L and 4.8 g/L, respectively (Table 1). The results of acetate consumption and lipid content from cultures on pure acetate solution elucidated that increasing acetate concentration within the experimental range benefited the fungal growth and lipid accumulation.

Meanwhile, fungal culture on acetate from AD showed that there was still 2.34 g/L acetate remained in the fermentation broth after 4 days culture with an initial acetate concentration of 4.34 g/L (Table 1). The lipid concentration was 0.03 g/L, and corresponding lipid conversion was 5.8% of the theoretical conversion. Compared with the cultures on pure acetate, consumption of acetate from AD was much slower (Fig. 3), and the lipid conversion and concentration were also lower (Table 1). The inferior performance of *M. isabellina* on the acetate solution from AD might be caused by some other compounds such as ethanol and propionic acids etc. that had inhibitory effects on the fungus. Thorough investigation is needed to delineate a clear analysis of the AD effluent components and their effect on the fungal fermentation.

Table 1. Lipid accumulation from pure acetate and AD effluent by *M.isabellina*

Substrates	Initial acetate concentration (g/L)	Final acetate concentration (g/L)	Lipid (g/L)	Lipid content in fungal biomass (w/w%)	Conversion (% of the theoretical value)*
Pure acetate	2.34	0	0.05	3.75	7.5
	4.8	0	0.15	9.93	10.9
	7.11	0.25	0.23	13.44	11.4
Acetate from AD	4.34	2.5	0.03	3.44	5.8

*: The theoretical conversion of lipid on acetate was 0.29 g lipid/g acetate

Engineered E. coli free fatty acid accumulation from acetic acid as sole carbon source

Figure 4 demonstrated the comparison of acetate utilization between wild-type *E. coli* and engineered *E. coli* with ace gene. It was apparent that wild-type *E. coli* had almost no capability to utilize acetate to accumulate a large amount of bacterial biomass. With expressing ace gene, the engineered *E. coli* significantly improved the efficiency of acetate utilization to accumulate biomass. In 14 days culture, 3.4 g/L acetate has been consumed to accumulate 0.88 g/L bacterial biomass (Fig. 4).

Table 2 showed that the engineered *E. Coli* with overexpression of both *ace* gene and *TesA* gene accumulated 0.06 g fatty acids/g acetate from the culture on pure acetate. In contrast, the same strain (at high OD_{600}= ~3) was able to utilize the acetate solution from AD, and produced 0.09 g fatty acid /g acetate. The conversions of the cultures on pure acetate and acetate from AD were 19% and 32% of the theoretical value, respectively (Table 2). The GC-MS analysis showed that the engineered *E.coli* strain produced mostly medium-chain fatty acids (C8-C16), which could be potentially used to make high quality biodiesel fuel.

Compared to fungal lipid accumulation from acetate, engineered *E. coli* demonstrated superior performance on acetate utilization efficiency. The conversions on pure acetate and acetate from AD were approximately twice and five times higher, respectively, than corresponding fungal cultures (Tables 1 and 2). Interestingly,

during fungal lipid accumulation acetate solution from AD had a negative impact on fungal lipid accumulation, while the same solution from AD had a positive impact on engineered *E. coli* fatty acid accumulation. The yield and conversion of acetate from AD were much higher than the use of pure acetate (Table 2). In-depth investigations are needed to further investigate the utilization of unidentified carbon substrates in the AD effluent by the engineered strain.

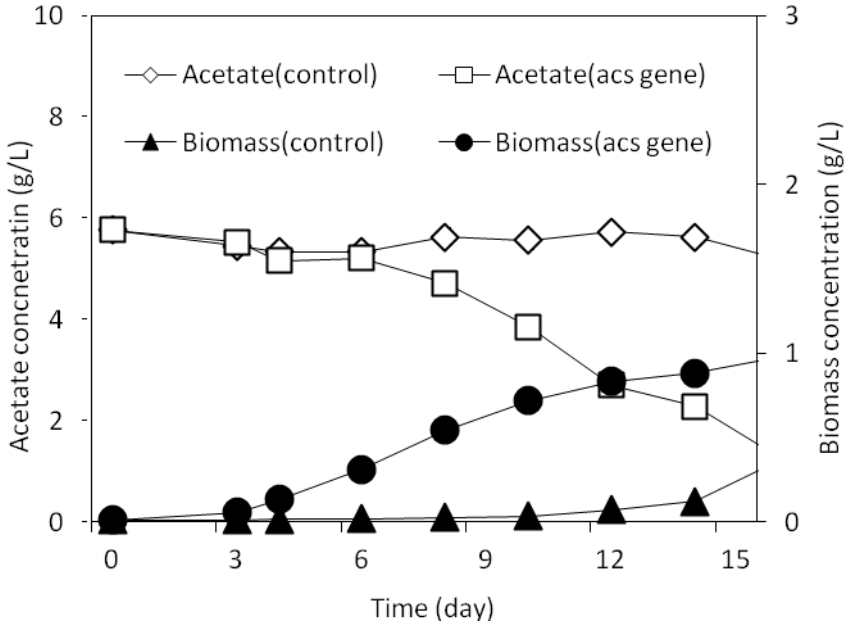

Figure 4. Acetic acid utilization by wild-type *E. coli* and engineered *E. coli* with acs gene (overexpress of acetyl-CoA synthase) in aerobic culture (37 ^{o}C). The culture medium contained M9 salts, pure acetate and 2 g/L yeast extract.

Table 2. The composition of fatty acids from engineered *E. coli* cultured from pure acetate and acetate from anaerobic digestion

Substrates	Initial acetate concentration (g/L)	Final acetate concentration (g/L)	lipid (g/L)	Fatty acid yield (g /g acetate)	Conversion (% of the theoretical value)*
Pure acetate	10	0	0.55	0.06	19
Acetate from AD	2	0	0.18	0.09	32

*: The theoretical conversion of lipid on acetate was 0.29 g lipid/g acetate

CONCLUSION

With the high annual yield and large amount of organic matters, sewage sludge is gaining more and more attention around the world as a promising source for value-added fuels/chemical production. Several treatment processes of sewage sludge have been researched and developed and applied to produce biodiesel (Kwon et al. 2012). In this study, we have combined the anaerobic digestion and microbial biotransformation to convert sewage sludge directly to high-quality microbial lipids (compared to the lipid existing in the sewage sludge) and prepare the feedstock for advanced biodiesel or biojet production. Anaerobic Digestion process on sewage sludge was optimized to accumulate acetate. The results indicated that inhibitor control was a more favorable strategy than pH control for acetate accumulation. After AD treatment of sewage sludge for acetate

production, both wild type *M.isabellina* and engineered *E.coli* demonstrated capabilities to utilize the acetate from AD waste as carbon source for lipid accumulation. Moreover, engineered *E. coli* demonstrated superior performance on utilization of the acetate from AD for bacterial lipid accumulation. This study not only demonstrates a practical way to combine AD process and microbial fermentation, but also indicates a promising alternative for further utilization of AD effluent to economically produce biodiesels and biojet. Our integrated system will provide a platform that can be extended to produce other types of value added chemicals in a sustainable and economically viable manner.

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