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Research article

Rumen fluid fermentation for enhancement of hydrolysis and acidification of grass clipping

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ABSTRACT

Rumen fluid, formed in rumen of ruminants, includes a complex microbial population of bacteria, protozoa, fungi and archaea, and has high ability to degrade lignocellulosic biomass. In this study, rumen fluid was used to ferment grass clipping for enhancing the hydrolysis and acidification of organic matters. Results showed that strict anaerobic condition, higher grass clipping content and smaller particle size of grass clipping were beneficial to the hydrolysis and acidification of organics. The increase of SCOD and total VFA concentration respectively reached 24.9 and 10.2 g/L with a suitable grass clipping content of 5%, a particle size < 0.150 mm, and a fermentation time of 48 h. The VFA production was mainly attributed to the degradation of cellulose and hemicellulose with a total solid reduction of 55.7%. *Firmicutes* and *Fibrobacteres* were the major contributors to the degradation of cellulose and hemicellulose. The activity of carboxymethyl cellulose enzyme (CMCase), cellobiase and xylanase reached 0.027, 0.176 and 0.180 U/ml, respectively. The rumen fluid microorganisms successfully enhanced the hydrolysis and acidification of grass clipping.

1. Introduction

Lignocellulosic biomass, such as agricultural residuals and a part of municipal wastes, is an abundant organic resource (Zheng et al., 2014). Large quantities of lignocellulosic residues have been accumulated from agriculture (corn stover, rice straw, etc.), forestry (woods, branches, foliage, etc.), energy crops (switch grass, yellow poplar, etc.), municipal green (grass, shrubs, etc.), and other sources (Chen et al., 2017). Abundant feedstock can be used for sustainable production of biofuels and bioproducts, such as biogas, bioethanol, bio-oil, volatile fatty acids (VFAs) and saccharides. Grass, as an abundant renewable lignocellulosic biomass, has enormous significant potential for this application due to its fast growth, large production, renewability and so on (Nizami and Murphy, 2010; Tao et al., 2018; Yu et al., 2014). Grass silage has been considered as an important feedstock or co-substrate for anaerobic digestion in Germany (Yu et al., 2014).

VFAs, as bioproducts, are short-chain fatty acids consisting of six or fewer carbon atoms, which can be distilled at atmospheric pressure (Lee et al., 2014). VFAs not only are used for the production of methane during anaerobic digestion process, but also have other extensive applications such as in the production of bioplastics and bioenergy, and biological removal of nutrients from wastewater. VFA production from different lignocellulosic biomass has drawn increasing attention in recent years (Guo et al., 2011; Park et al., 2015). The amounts of VFAs produced by rumen bacteria from rice straw and Japanese cedar were 212.28 and 65.40 mg/g total solid (TS), respectively (Agematu et al., 2017). Yu et al. (2014) pretreated lawn grass by soaking in aqueous ammonia for enhancing VFA production, and the VFAs yield reached 175.6–263.4 mg/g TS.

In general, the VFA production from lignocellulosic biomass is an anaerobic process involving hydrolysis and acidification (Lee et al., 2014). However, the hydrolysis of lignocellulose biomass often becomes the rate-limiting step during traditional fermentation (Jeihanipour et al., 2011; Li et al., 2011), which is mainly due to two reasons. The first reason is that the interactions of lignocellulosic biomass composition (cellulose, hemicellulose and lignin) create a highly resistant and recalcitrant biomass structure, therefore, various pretreatment methods have been developed to destroy the lignocellulosic structure in order to improve the hydrolysis rate. The second reason is that the microorganisms of hydrolysis and acidification during traditional fermentation have low hydrolysis rate. The corresponding solution is to use more efficient microorganisms for degrading

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lignocellulosic biomass in the hydrolysis and acidification step.

Rumen fluid, formed in the fore-stomach (rumen) of cows, includes a complex microbial population of bacteria, protozoa, fungi and archaea, which exhibits higher ability to degrade lignocellulosic biomass than other normal anaerobic microorganisms (Yue et al., 2013). Therefore, the application of rumen fluid has been recently examined for potential use in the biodegradation of lignocellulosic biomass (Yue et al., 2013). The rumen microorganism addition into an anaerobic digestion system of peanut hull resulted in an effective degradation of structural components (cellulose, hemicellulose and lignin) (Dahunsi et al., 2017). Wall et al. (2015) used rumen fluid for the anaerobic digestion of high lignocellulose grass silage, and the results demonstrated that the methane yield reached 371 ml CH_4/g volatile solid (VS) with an increase of VFA concentration. Zhang et al. (2016) pretreated rice straw with the rumen fluid at 39 °C for 24 h before anaerobic digestion, and the biogas production increased by 67%, the methane yield increased by 83%, and the digestion time decreased by 40%. These findings showed that rumen fluid was a promising inoculum for enhancing lignocellulose degradation. However, only in a few studies, the rumen fluid was applied to improve the hydrolysis and acidification of lignocellulose for VFA production.

On the other hand, the researches of rumen fluid mainly focused on nutritional uptake of animals. Microorganisms and enzymes in rumen were used to degrade organic matters (cellulose, hemicellulose, protein, starch et al.), and enzymes played an important role to the degradation of organic matters (Sauer et al., 2012). The corresponding degrading enzymes include cellulase, α-glucosidase and β-glucosidase, xylanase, protease, α-amylase, urease and so on (Liu et al., 2014). However, few studies involved the function of microorganisms and enzymes in the hydrolysis and acidification of lignocellulose for VFA production *in vitro*.

In this paper, rumen fluid fermentation was used as a green technology for enhancing the hydrolysis and acidification of grass clipping, aiming to improve the VFA production. This paper also assessed the feasibility of rumen fluid fermentation and investigated several influencing factors including dissolved oxygen (DO), grass clipping content and particle size. In addition, the microorganisms and enzymatic activity and chemical composition of grass clipping during fermentation were analyzed to study the fermentation mechanisms.

2. Materials and methods

2.1. Raw materials

Grass clipping of lawn grass was collected from the campus of Beijing Forestry University, China. The grass clipping was cleaned and dried at room temperature for one week, and then milled to a particle size of 0.425–0.850 mm, 0.150–0.425 mm, and < 0.150 mm. The triturate of grass clipping was dried at 65 °C for 12 h, and then stored in sealed plastic bags at room temperature. The VS contents of above grass clipping were 0.854 g/g dry substrate.

2.2. Rumen fluid

Rumen fluid was collected from the fistulated beef cattle in the Beef Cattle Research Center of China Agricultural University, and was transferred into vacuum thermos bottle to keep the activity of rumen microorganisms. The rumen fluid was filtered through four-layer sterile gauze in order to remove large particles and no-degraded substrates (Li et al., 2017; Wall et al., 2015). The filtered rumen fluid was then characterized, and the pH was 6.48, the SCOD was 12865 mg/L, and the TS and VS were 24.2 and 15.2 g/L, respectively.

2.3. Rumen fluid fermentation of grass clipping

100 ml serum bottles were used as the fermentation reactors. 0.7, 2.1 or 3.5 g grass clipping and 70 ml rumen fluid were added into different reactors and formulated to 1%, 3% and 5% (*w/v*) mixture, respectively, filling with nitrogen to keep the anaerobic environment and sealing with a rubber stopper. The serum bottles were shaken at 39 °C for 72 h in an air bath thermostat oscillator (THZ-82B, Jintan shenwei equipment Co., Ltd, China) with a speed of 100 r/min. 0.7 g grass clipping and 70 ml deionized water were added into reactors and reacted under the same conditions, as the control. The experiments were carried out in a way to avoid volume change of samples. For each reaction condition, 18 experiments were carried out. The sampling time was 4, 8, 16, 24, 48 and 72 h, and three serum bottles were taken out every time for sampling. The samples were centrifuged at 8000 r/min for 10 min by a refrigerated high-speed centrifuge (3H16RI, Hunan Hersxi instrument & equipment Co., Ltd, China). The supernatant was filtered through 0.45 mm filter membrane after pH measurement. The filtrate was used for the determination of VFAs, SCOD and enzymatic activity. The solid residue produced by centrifugation was washed with deionized water, dried at 105 °C for at least 4 h in a drying cabinet, and then stored in sealed plastic bags at room temperature for the measurement of chemical composition.

2.4. Rumen fluid fermentation of grass clipping in micro-aerobic condition

2.1 g grass clipping was mixed with 70 ml rumen fluid as 3% (*w/v*) in 100 ml serum bottles, filling with nitrogen to remove oxygen and sealed with a rubber stopper. The test was divided into 5 groups, and 10, 20, 30, 40 μl oxygen was injected into liquid fraction of four groups to obtain a dissolved oxygen of 0.2, 0.4, 0.6 and 0.8 mg/L, respectively. The last one without oxygen injection was considered as the control. The serum bottles were shaken at 39 °C for 72 h in the air bath thermostat oscillator (100 r/min). The followed procedure was similar to that mentioned in rumen fluid fermentation of grass clipping.

2.5. Analysis methods

The chemical composition of raw and fermented grass clipping was analyzed by a fiber analyzer (A200i, ANKOM, Macedon NY14502, USA). The determination of VFAs was conducted by a gas chromatography (GC-2018, Schimadzu (China) Co., China). The concentration of SCOD was determined by a water quality analyzer (5B-1B, Lian-hua Tech Co., China). The pH was measured by a pH meter (PHS-3E, Shanghai INESA Scientific Instrument Co., China).

To analyze the composition of microbial community during rumen fluid fermentation of grass clipping, samples were collected at different time of rumen fluid fermentation. Then, all samples were stored at −80 °C before DNA extraction. DNA extraction was performed using the E.Z.N.A. soil DNA extraction kit (Omega Bio-tek, Norcross, GA, U.S.). PCR reactions were performed in triplicate 20 μl mixture containing 4 μl 5 × FastPfu Buffer, 2 μl 2.5 mmol/L dNTPs, 0.8 μl primer (5 μmol/L), 0.4 μl FastPfu Polymerase and 10 ng template DNA. After purification and quantification of PCR products, amplicon sequencing was conducted using an Illumina MiSeq PE250 platform (Shanghai Majorbio Bio-pharm Technology Co. Ltd., China).

The activities of carboxymethyl cellulose enzyme (CMCase), cellobiase and xylanase were measured as follows. The reaction mixtures for the determination of CMCase, cellobiase and xylanase were incubated at 50 °C for 30, 30 and 10 min, respectively, and then boiled in water bath for 5 min after adding 1.5 ml DNS solution for stopping the reaction (Li et al., 2017; Liu et al., 2014). For measuring the activities of CMCase, the reaction mixture contained 0.5 ml 1.0% carboxymethyl cellulose solution, which was dissolved in 0.5 mol/L sodium citrate buffer solution (pH 4.8) and 0.5 ml filtered samples or deionized water (as control). For cellobiase, the reaction mixture contained 0.5 ml 15 mmol/L cellobiose solution, which was dissolved in 0.5 mol/L sodium citrate buffer solution (pH 4.8) and 0.5 ml filtered samples or deionized water (as control). For xylanase, the reaction mixture