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Use of Iodoform to Improve Lactic Acid Production in the Biomass Ensilage Conversion System

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Abstract. *Iodoform, an iodine-containing compound used in antiseptic applications, has been found to be effective at selectivity inhibiting certain microbial populations. Application of iodoform in a hybrid fermentation system was investigated to determine the potential for increased lactic acid production by inhibiting undesirable microbes which can metabolize lactic acid. Iodoform treatment rates of 0, 0.03, 0.06, 0.11, and 0.23 g/kg dry matter (DM) were applied to a swine manure-corn stover substrate, containing 60 % manure, adjusted to 65 % moisture on a wet basis and ensiled for 0, 1, 7, and 21 days. A hemicellulase-cellulase enzyme mixture was also applied to all samples at a*

rate of 5 and 12.5 IU/g DM of hemicellulase and cellulase activity, respectively. Samples were analyzed for pH, water soluble carbohydrates, and organic acids. A substantial decrease in pH was observed in all treatments, but none of the treatments reached a pH of 4.5, which is sufficient for stable storage of corn stover biomass at 65 % moisture. Lactic and acetic acid production was increased with application of iodoform at 0.23 g/kg DM. Iodoform was also found to inhibit butyric fermentation, with a rate of 0.23 g/kg DM determined to be appropriate. Overall, iodoform can improve fermentation in the biomass ensilage conversion system by improving lactic acid production and inhibiting butyric fermentation.

Keywords. Iodoform, manure, lactic acid, biomass, ensilage, fermentation, conversion

Introduction

Corn stover is the residue remaining after harvest of corn grain. It is considered one of the most readily available and abundant biomass feedstocks (NRC, 2000) and has gained much attention as a potential lignocellulosic biomass for producing ethanol (Wyman, 2003). An estimated 200 million dry metric tons of corn stover remain after harvest each year (Glassner et al., 1999). Gallagher and Johnson (1995) place corn stover availability at 100 million dry metric tons per year, which assumes that 30 percent of the residues are left on the soil surface. More conservative estimates by the Chief Executive Assistance (2000) and the USDA-DOE (2005) place the amount of corn stover that can be collected yearly at 73 and 75 million dry metric tons, respectively.

Livestock manure is a largely untapped source of agricultural residues for biomass conversion. A majority of livestock manure is land-applied to provide nutrients for crop growth, but in locations where the land area required for application is greater than the land area available for application, there is a definite problem. Utilizing these excess manure stocks for conversion could present a possible solution. Manure contains a high concentration of available nutrients which may otherwise need to be supplemented in a microbial conversion which utilizes a lignocellulose substrate (ASAE *Standards*, 2005). Manure produced from large concentrated animal feeding operations (CAFOs) is fairly concentrated in nature and would have lower collection and transportation costs as compared to manure generated by smaller livestock production facilities. Because of the low dry matter content of manure, (approx. 10 %), the relative amount of substrate available for conversion greatly limits its use in many conversion processes. Livestock manure as a biomass resource may be best used as an amendment to high lignocellulosic residues.

Conversion Technologies

Fermentation and enzymatic hydrolysis are thought to be the most flexible biochemical means to produce biobased industrial products (NRC, 2000) and transportation fuels (NRC, 1999) from lignocellulosic materials. Most conventional fermentation processes involve liquid systems with submerged or surface-adhered cultures, but solid-state systems offer many advantages. Because large volumes of water are not needed in solid-state systems, fermentation vessels are smaller, less energy is required, higher product concentrations can be reached, and the cost of product separation from the aqueous phase is reduced (Bothast et al., 1989). Ensilage, an anaerobic solid-state fermentation process traditionally used by ruminant producers to preserve high fiber feedstuffs for year-round use, has gained some attention not only as biomass storage method, but as a low-cost technology to produce multiple organic chemicals from biomass.

Ensilage is characterized as primarily a lactic acid fermentation process. During the initial stage of fermentation, excess oxygen is consumed producing an anaerobic environment. During the second stage, typically occurring from one day to three weeks, soluble carbohydrates are converted to lactic acid, acetic acid, ethanol, mannitol, acetaldehyde, and carbon dioxide by homofermentative and heterofermentative lactic acid bacteria (Roberts, 1995). This period is characterized by a significant decrease in pH. After three weeks, significant acetic and lactic acid accumulation result in pH declining to a level which inhibits further microbial growth and the silage is considered to be stable. The pH level which is sufficient for storage is dependent on many parameters, including substrate composition and ensiling conditions (McDonald et al., 1991). A range from pH 4.2 to 4.5 has been suggested for producing good quality silage (Woolford, 1984). Ren et al. (2004) found that reduction of pH to 4.5 guaranteed preservation of corn stover for a minimum of six months. If a satisfactory pH level is not reached in sufficient

time, clostridial spoilage can occur. Clostridia bacteria convert sugars and lactic acid to butyric acid, propionic acid, carbon dioxide, and hydrogen gas (Moser, 1980; Pitt, 1990). Butyric fermentation is considered to be detrimental to the ensilage process because butyric acid is a weak acid for preserving silage and considerable losses in energy from the silage occurs (>20%) (Jaster, 1995).

Biomass Ensilage Conversion System

The biomass ensilage conversion (BEC) system is an integrated process which takes advantage of both the fermentation and storage capabilities of ensilage (figure 1). The primary goals of the BEC system are production of organic acids, mainly lactic acid and acetic acid, and pretreatment and storage of high lignocellulosic materials to be used for downstream processing, such as ethanol fermentation (Murphy, 2006a). The BEC system has been primarily developed for use of corn stover as the substrate material, but this system can be configured for other high-moisture lignocellulosic materials.

Iodoform is an iodine containing compound, which is used in antiseptic applications in the medical and veterinary medicine fields (Windholz et al., 1983). Iodoform has been found to selectively inhibit the activity of methanogenic bacteria in a mixed culture with acid-forming bacteria (Aiello-Mazzarri, 2006). Iodoform has been used extensively in anaerobic digestion studies, but not applied in ensilage systems. Investigation of iodoform to improve lactic acid yields by inhibiting unfavorable silage microbes in a hybrid ensilage system is needed.

The objectives of this experiment were to determine the effects of iodoform on lactic acid and volatile fatty acid production during the fermentation phase of the BEC system and to determine possible application rates for its use.

Materials and Methods

An experiment was conducted to determine the effect of iodoform treatment on lactic acid and volatile fatty acid production in corn stover-manure substrate ensiled for a period of 21 days. Iodoform application rates of 0, 0.03, 0.06, 0.11, and 0.23 g/kg dry matter (DM) and ensilage periods of 0, 1, 7, and 21 days were evaluated using a split-plot design with three replications. Iodoform treatments were applied to whole samples and ensilage time was applied to subsequent sub-samples.

Treatment Procedure

Large round bales of corn stover (hybrid Pioneer 34H31 produced during the 2004 cropping season) were obtained from a livestock and grain producer near State Center, Iowa. The whole-plant stover was fractionated using a portable shredder (MTD Products Inc., Valley City, OH) equipped with a screen having 12 mm by 80 mm slots to produce a smaller, more uniform particle size for improved fermentation (Ren et al., 2004). The initial moisture content of the corn stover was 15 % on a wet basis (w.b.), as determined by drying 100 g of material at 60 °C in a forced air oven for 72 h (ASAE *Standards*, 2003).

Swine manure was acquired from the Iowa State University Bilsland Swine Research Farm near Madrid, Iowa. The manure was obtained from a storage pit located beneath a confinement building for finishing swine which is pumped into a lagoon every two weeks. The manure contained approximately 10 % solids (w.b.), which is similar to the average solids content for finishing swine manure (ASAE *Standards*, 2005). Solids content of the manure was determined by drying 100 g of sample at 103 °C in a forced air oven for 4 h.

Corn stover and manure were blended to produce a mixture containing 60% manure (w.b.), with the mixture being simultaneously adjusted to 65 % moisture (w.b.) by addition of water. This manure level is the maximum approximate content for adjusting the corn stover to an optimum 65 % moisture content (Hoglund, 1964) for ensiling with only minimal water addition. A hemicellulase-cellulase enzyme mixture, Multifect A40 (Genecor, Cedar Rapids, IA), was applied to all samples at a rate of 5 and 12.5 IU/g DM of hemicellulase and cellulase activity, respectively, to ensure adequate sugars for fermentation (Ren et al., 2004; Richard et al., 2002). An iodoform solution was prepared by dissolving 1g of iodoform into 60 mL ethanol and treatments were adjusted to the same ethanol content as the highest iodoform rate. Iodoform treatments were applied to triplicate 2-kg samples and separated into equal 500-g subsamples, corresponding to each of the four ensilage periods. Subsamples were then vacuum-sealed in polyethylene bags and incubated for 0, 1, 7, or 21 days at 37 ºC.

Laboratory Analyses

After ensiling, samples were removed from the polyethylene bags, mixed thoroughly, and analyzed for pH, dry matter, water soluble carbohydrates (WSC), and organic acids.

Dry matter of the subsamples was determined by drying 100 g of material at 60°C in a forced air oven for 72 h (ASAE *Standards*, 2003). Dried samples were ground using a Wiley mill (Thomas Scientific Inc., Swedesboro, NJ) fitted with a 1-mm sieve and used for water soluble carbohydrates analysis. Dry matter was also determined for ground samples by drying 1 g of sample at 103 °C in a forced air oven for 4 h to make moisture corrections for water soluble carbohydrates. pH was measured using a pH electrode. Samples were prepared with a 10:1 mass dilution (H_2O: sample) and allowed to stand for 30 min prior to measurement.

Water soluble carbohydrates were determined using a modification of the method described by Guiragossian et al. (1977). Water extracts were prepared by shaking 0.25 g of the ground material with 100 mL of distilled water for 30 min. Extracts were then filtered through #54 filter paper. To an aliquot of the filtrate, 5 % phenol and sulfuric acid was added and the solution's absorbance was measured at 490 nm using a UV-1601 spectrophotometer (Shimadzu Corp., Columbia, MD) equipped with a rectangular 10 mm light path cell. Sample values were calculated from a standard calibration curve prepared using equimolar concentrations of glucose and xylose to determine carbohydrate levels.

Organic acid concentrations were analyzed using a method similar to that described by Moore et al. (1985). Water extracts were prepared by shaking 50 g of sample with 200 mL of distilled water for 4 h, refrigerating at 5 °C for 20 h, and filtering through four layers of cheese cloth. An aliquot of the filtrate was acidified with 25 % metaphosphoric acid and centrifuged for 15 min at 15,000 rpm. The resulting supernatant was collected and analyzed using gas-liquid chromatography. Separations of acetate, propionate, butyrate, and iso-butyrate were made using an SP-1200/H_3PO_4 column (Sigma-Aldrich Co., St. Louis, MO) operated at 120 °C using N_2 as the carrier gas at a flow rate of 30 mL/min with an injection block temperature of 170 °C. Quantification of the acids was done using a flame ionization detector operating at 180 °C. Lactate concentrations were determined using a similar procedure. Separation was done using an SP-1000/H_3PO_4 column (Sigma-Aldrich Co., St. Louis, MO) with the initial oven temperature set at 100 °C for 1 min and increased to 120 °C at a rate of 10 °C/min. Prior to injection, lactate was methylated by combining 2 mL of filtrate, 4 mL of methanol, and 0.8 mL of 50 % aqueous H_2S in a test tube and heating for 30 min. at 60 °C (Supelco, 1998). After cooling, 2 mL of water and 2 mL methylene chloride were added to the test tube and the resulting bottom layer of methylene chloride was sampled. All other operating conditions were the same as described above. Total organic acid content was calculated as the summation of lactate, acetate, propionate, butyrate, and iso-butyrate values.

Statistical Analysis

Statistical analysis was done using Statistical Analysis Systems software (SAS, 2003). The general linear model (GLM) procedure was used to determine the statistical significance of treatment effects and mean comparisons were made using an appropriate least significant difference (LSD). Differences among treatment means were determined to be significant at $p \leq 0.05$.

Results and Discussion

pH

Rapid acidification occurred with all iodoform treatment levels during the first day of ensiling, with smaller pH changes occurring between day 1 and 21 (figure 2). Nevertheless, during the 21-day fermentation period, none of the iodoform treatments progressed to a level lower than pH 4.5, which is considered adequate for stable storage. The absence of sufficiently low pH values is likely a result of the high buffering capacity of the manure compared to corn stover (Murphy, 2006b). The buffering capacity of corn stover and manure was 60 and 280 mequiv./kg DM, respectively, as determined by the method of Playne and McDonald (1966). Further research is needed to determine acceptable levels of manure which can be used in this hybrid ensilage system.

Water Soluble Carbohydrates

Iodoform concentration was found not to have an effect on water soluble carbohydrate levels, indicating that iodoform does not impact enzymatic hydrolysis of hemicellulose and cellulose to fermentable sugars. Average WSC levels across all treatments decreased from an initial level of 1.7 % DM during the first day of ensiling and maintained a level of approximately 1.4 % DM throughout the remainder the 21-day ensilage period (figure 3).

Organic Acids

An increase in total organic acid concentration of several percentage points DM was observed across all treatments during the first day of ensiling, with smaller changes occurring over the subsequent fermentation period (figure 4). At day 1, the 0.06, 0.11, and 0.23 g/kg treatments produced concentrations of 5.1, 4.6, and 4.6 % DM, respectively, which were higher than the control treatment of 3.7 % DM. Concentrations in the 0.23 g/kg treatment were higher than the other treatments the remainder the ensilage period, increasing to 5.9 % DM at day 7 and falling to 5.5 % DM at day 21. By day 21, concentrations in all other iodoform treatments had dropped to levels similar to the control. Losses in organic acid may be attributed to the evolution of hydrogen gas and carbon dioxide during butyric fermentation (McGechan, 1990).

Organic acid production was dominated by lactate during the initial day of fermentation and by butyrate the remainder of the ensilage period. The interaction effect of ensiling time and iodoform rate was found to be significant for concentrations of lactate, butyrate, and acetate (figures 5, 6, and 7).

Increases in lactate were observed in all treatments at day 1, with the iodoform applications of 0.03, 0.06, 0.11, and 0.23 g/kg DM reaching levels of 0.9, 1.3, 2.1, and 2.3 % DM compared to 0.6 % DM for the control (figure 5). By day 7 a majority of the lactate was consumed in the control and the 0.03 and 0.06 g/kg DM treatments and remained near 0 % DM the remainder of the trial. A sizable decrease in lactate was also observed in the 0.11 g/kg treatment at day 7,

which continued to drop, reaching near control levels by day 21. Lactate concentration continued to increase to 2.8 % DM at day 7 in the 0.23 g/kg DM rate, but fell dramatically to 1.5 % DM by day 21.

Decreases in lactate after day 1 were correlated with increases in butyrate thereafter (figure 6). At day 1, the 0.11 and 0.23 g/kg treatments produced a butyrate concentration of 0.4 % DM, which was lower than the 1.0 % DM produced by the other treatments. With the exception of the 0.23 kg/g DM rate, an increase in butyrate concentration was observed in all treatments between day 1 and 7, with the control producing the highest concentration of 2.0 % DM. At day 21, the 0.06, 0.11, 0.23 g/kg DM rates produced a butyrate level of about 1.0 % DM which was lower than the 1.70 % DM level produced by the control and 0.03 g/kg treatments.

Acetate levels increased between 0.5 and 0.7 percentage points DM across all treatments during the 21-day ensilage period (figure 7). The 0.23 g/kg DM rate was the only treatment that consistently produced higher acetate levels than the control from day 1 to 21, producing an average increase of 0.6 percentage points DM.

Organic acid results indicate that lactate production was increased with iodoform rates of 0.11 and 0.23 g/kg DM during the first week of ensiling and acetate production was increased with an iodoform rate of 0.23 g/kg DM during the 21 days of ensiling. Treatments with the lower iodoform rates may also provide some increase in lactate production compared to the control, but because of the considerable fermentation of lactate to butyrate by clostridia bacteria, this could not be determined. Maximum lactate and acetate concentrations of 2.8 and 3.0 % DM, respectively, indicate considerable conversion of both hexose and pentose sugars by homofermentative and heterofermentative lactic acid bacteria (Woolford, 1984). However, the equivalent lactate concentration of approximately 1.0 % (w.b.) would most likely not be economically competitive with starch-based lactic acid production methods which can achieve concentrations over 10 % (Oh et al., 2005; Tsao et al., 1999).

The inhibitory effect of iodoform on butyric fermentation and presumably clostridia activity is dependent on the length of the fermentation period, the time required for the pH to drop below 4.5, and the rate of iodoform applied. For instance, in this experiment if the fermentation period was 1 day, an iodoform rate of 0.11 g/kg DM would be necessary; if the fermentation period was 7 days, a rate of 0.23 g/kg DM would be necessary. For this experiment, a rate of 0.23 g/kg DM was suitable for inhibiting butyric fermentation throughout the 21-day ensilage period. Butyrate production of 1.0 % DM was observed within the first day of ensiling, which is earlier in the fermentation phase than anticipated. Ren et al. (2004) observed butyrate levels greater than 1.0 % DM after 7 days of ensiling using a corn stover substrate with a hemicellulase and cellulase enzyme mixture. This earlier occurrence of butyric acid formation is likely a result of significant levels of clostridia which occur naturally in manure (McDonald et al., 1991).

Overall, an iodoform rate 0.23 g/kg DM would be suitable for application in the biomass ensilage conversion system to improve lactate and acetate yields, as well as inhibit butyric fermentation. Further investigation of rates greater than 0.23 g/kg DM would be valuable to determine an optimum level of iodoform to apply.

Conclusions

Substantial increases in lactic acid during ensiling were achieved with iodoform applied at rates exceeding 0.11 g/kg DM, with rates of 0.23 g/kg DM also producing a significant increase in acetic acid. Butyric fermentation was inhibited with application of iodoform, but the inhibition mechanism is dependent the iodoform concentration and the duration of the fermentation period during the ensilage process. Application of iodoform at a rate of 0.23 g/kg DM would be

appropriate for improving lactic and acetic acid yields and limiting secondary fermentation in an ensilage system for bioconversion. Due to the high concentration of manure used in the substrate (60 %) and resulting high buffering capacity compared to a corn stover substrate containing no manure, none of the treatments reached a pH that would be sufficient for long-term storage. These results indicate that iodoform can improve fermentation in the biomass ensilage conversion system by improving lactic acid production and inhibiting butyric fermentation.

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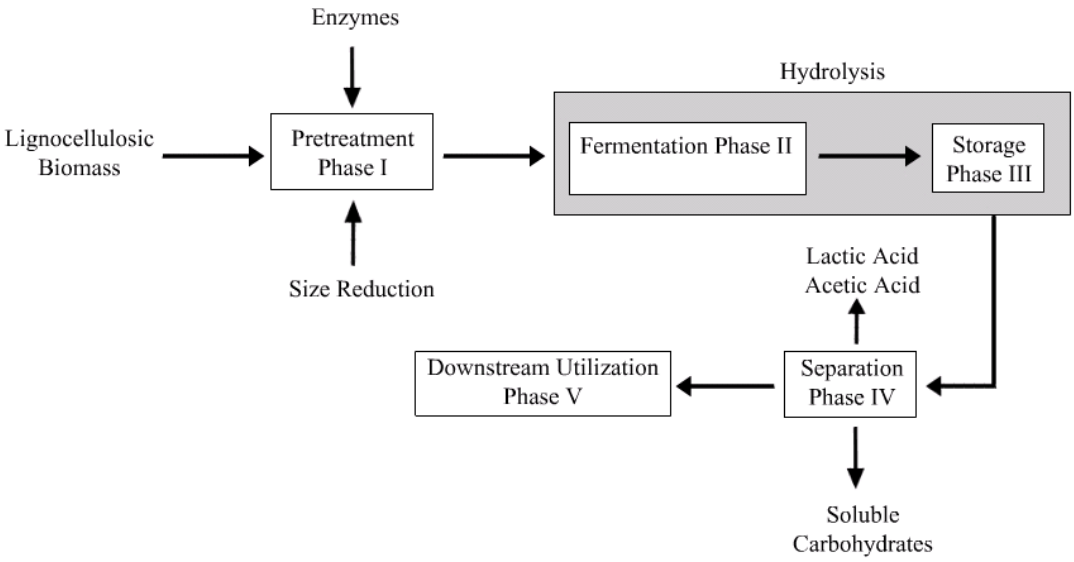

Figure 1. Biomass ensilage conversion (BEC) system schematic.

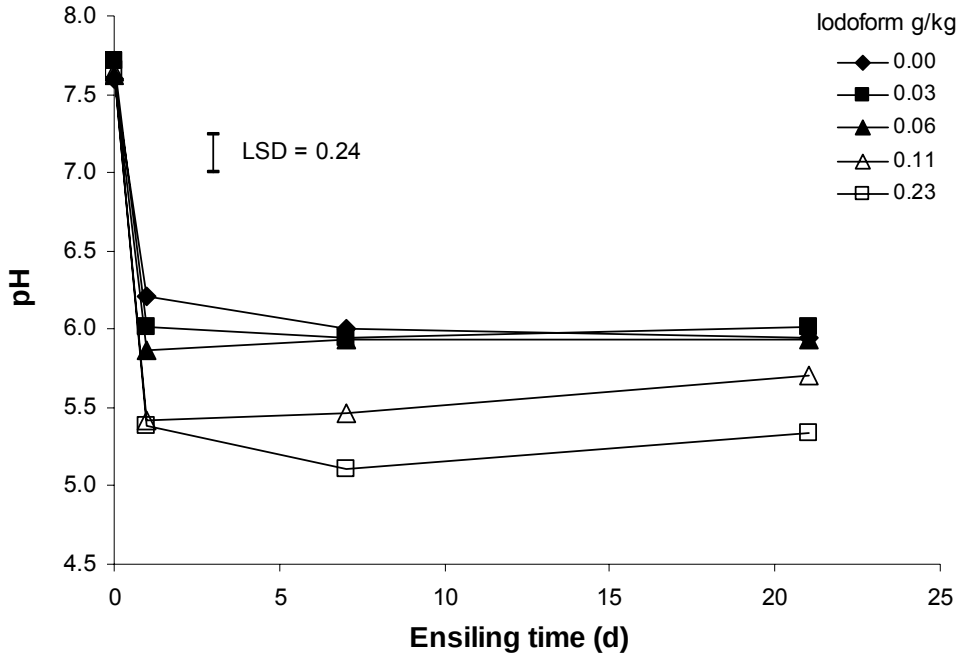

Figure 2. pH of corn stover-manure substrate treated with iodoform at 0, 0.03, 0.06, 0.11, and 0.23 g/kg DM at 0, 1, 7, and 21 days of ensiling (n=3). $LSD_{0.05}$ is appropriate for comparison of iodoform treatments within a time.

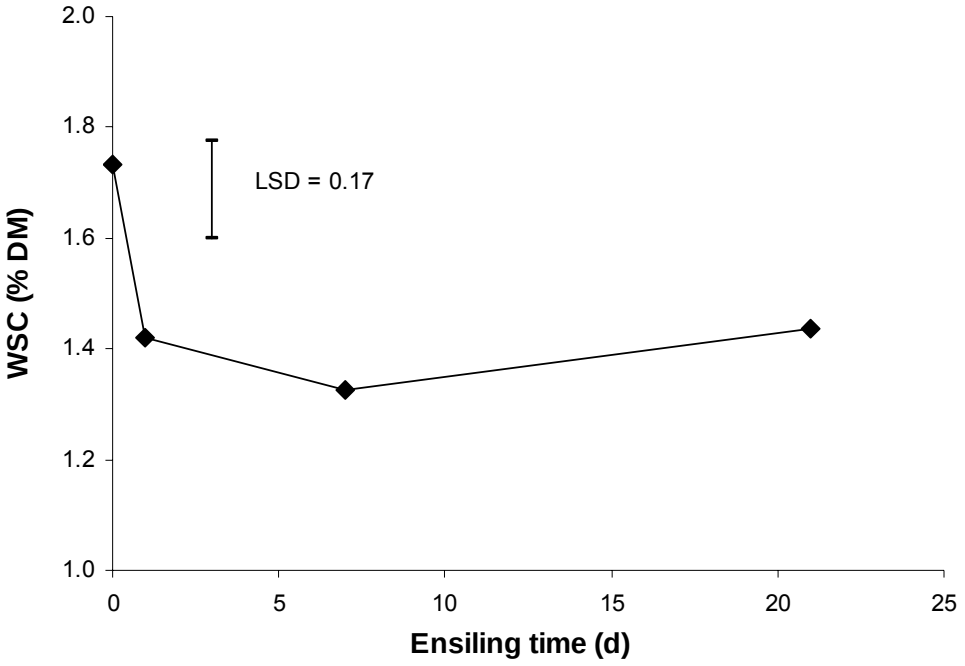

Figure 3. Average concentrations of water soluble carbohydrates (WSC) in corn stover-manure substrate treated with iodoform at 0 to 0.23 g/kg DM at 0, 1, 7, and 21 days of ensiling (n=15). $LSD_{0.05}$ is appropriate for comparison of ensiling times.

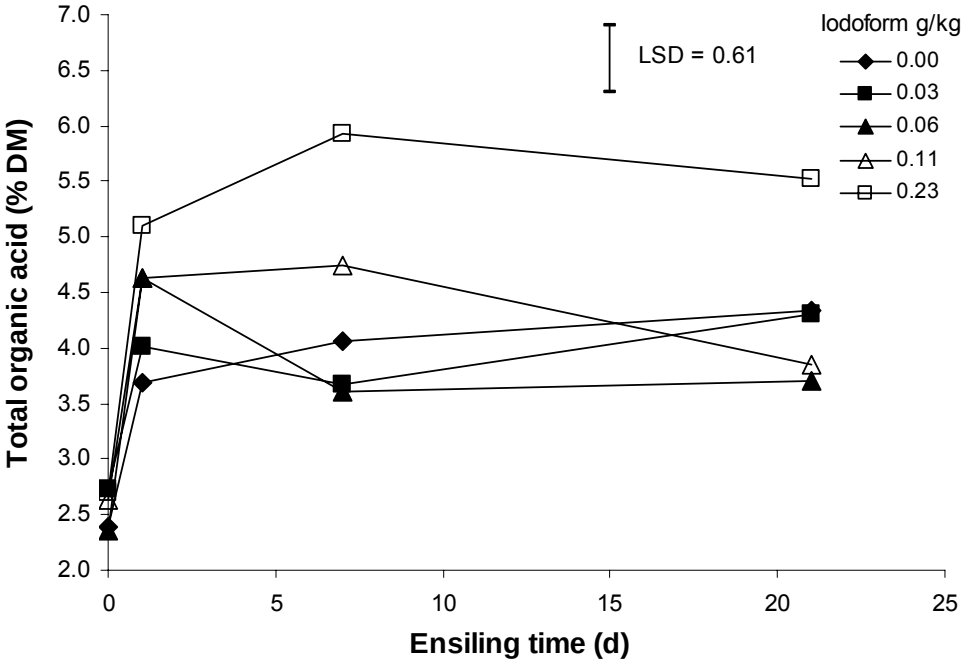

Figure 4. Concentrations of organic acid in corn stover-manure substrate treated with iodoform at 0, 0.03, 0.06, 0.11, and 0.23 g/kg DM at 0, 1, 7, and 21 days of ensiling (n=3). $LSD_{0.05}$ is appropriate for comparison of iodoform treatments within a time.

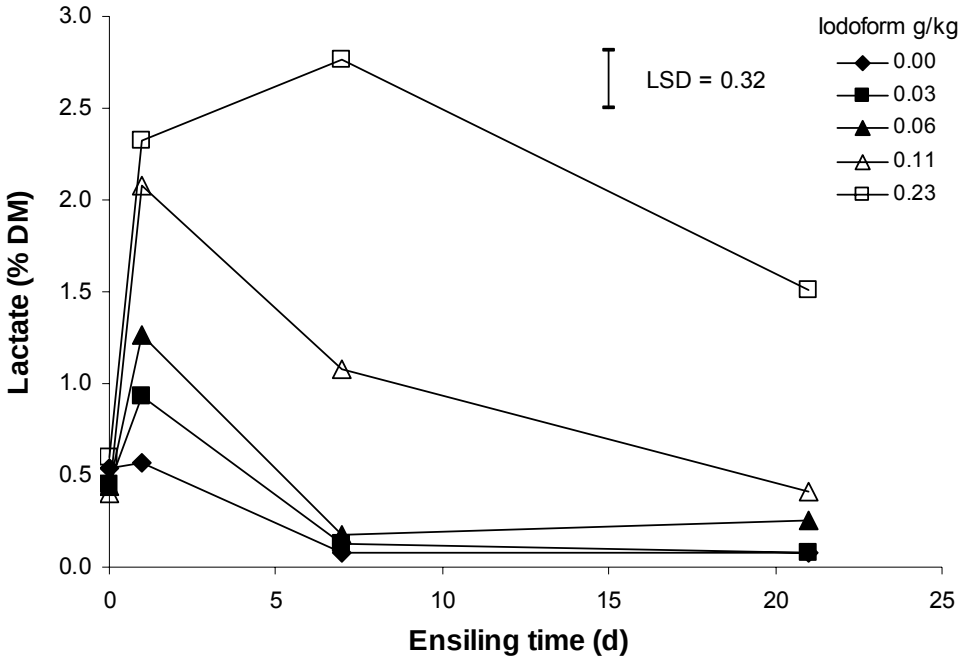

Figure 5. Concentrations of lactate in corn stover-manure substrate treated with iodoform at 0, 0.03, 0.06, 0.11, and 0.23 g/kg DM at 0, 1, 7, and 21 days of ensiling (n=3). $LSD_{0.05}$ is appropriate for comparison of iodoform treatments within a time.

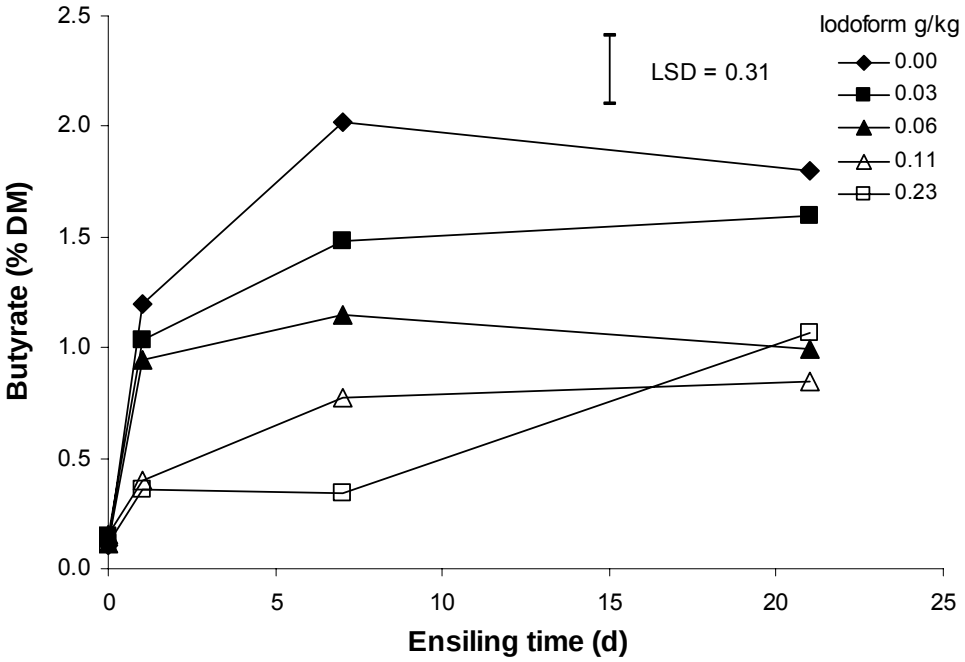

Figure 6. Concentrations of butyrate in corn stover-manure substrate treated with iodoform at 0, 0.03, 0.06, 0.11, and 0.23 g/kg DM at 0, 1, 7, and 21 days of ensiling (n=3). $LSD_{0.05}$ is appropriate for comparison of iodoform treatments within a time.

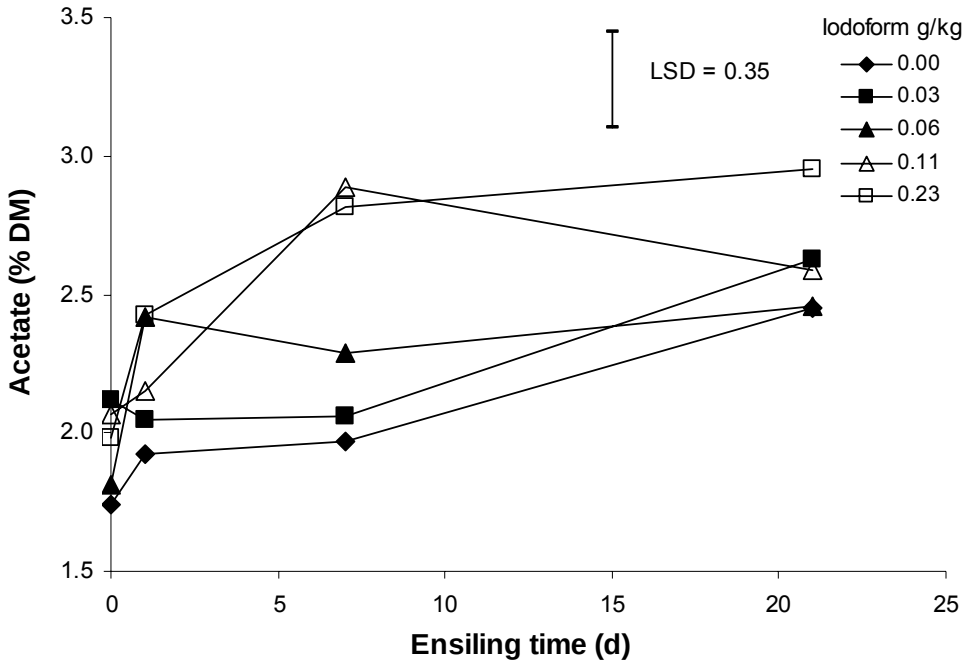

Figure 7. Concentrations of acetate in corn stover-manure substrate treated with iodoform at 0, 0.03, 0.06, 0.11, and 0.23 g/kg DM at 0, 1, 7, and 21 days of ensiling (n=3). $LSD_{0.05}$ is appropriate for comparison of iodoform treatments within a time.