

# Development of highly digestible animal feed from lignocellulosic biomass

## Part 1: Oxidative lime pretreatment (OLP) and ball milling of forage sorghum<sup>1</sup>

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**ABSTRACT:** To feed a growing population, alternative sources of animal feed (e.g., lignocellulose) are needed to replace grains (e.g., corn). Oxidative lime pretreatment (OLP) increases lignocellulose digestibility by removing lignin and hemicellulose acetyl content. Adding a mechanical pretreatment (e.g., ball milling) further improves digestibility. This study determines the effectiveness of OLP and ball milling to enhance the ruminant digestibility of lignocellulose. For forage sorghum, the 48-h in vitro TDN were 40, 64, and 84 g nutrients digested/100 g organic matter (OM) for raw, short-term OLP, and short-term OLP + ball milling, respectively. In terms of compositional changes, OLP increases NDF and decreases non-fiber

carbohydrate (NFC) and crude protein (CP), all of which would normally be associated with a decrease in digestibility. However, because OLP and ball milling beneficially change composition (lignin removal) and structural features (reduced crystallinity), digestibility actually increases. Although ball milling increases digestibility according to standard laboratory assays, it reduces particle size possibly allowing fine particles to escape from the rumen before they are digested, thus limiting its practical application. Nonetheless, this study indicates that mechanical pretreatment greatly increases digestibility, and therefore it is desirable to identify an effective mechanical treatment that retains fiber integrity.

**Key words:** ball milling, lignocellulose, lime pretreatment, ruminant animal feed

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## INTRODUCTION

Corn grain is heavily used as livestock feed because it is rich in highly digestible carbohydrates and proteins. Recently, the price of corn has increased to \$4 to \$7/bushel (2006 to 2016) from \$2 to \$3/bushel (1975 to 2005; USDA, 2015). As a consequence of high grain prices, the price of pork, poultry, beef, dairy products, and other agricultural commodities commonly increases as well. Furthermore, increases in demand for food and grain-based biofuels also place a steady upward pressure on corn prices (Mitchel, 2008).

For ruminants, it is possible to replace corn with lignocellulose, the most abundant organic material on

earth (Rajaratnam et al., 1989). The anaerobic fermentation that occurs in the rumen is extremely versatile and can digest not only non-fiber carbohydrates, but also fibrous cellulose and hemicellulose (Wang and McAllister, 2002). Unfortunately, because of its structural features (e.g., lignin content, hemicellulose acetyl content, and cellulose crystallinity), lignocellulose is highly recalcitrant and requires research to identify methods to increase its digestibility (Mosier et al., 2005). Chang and Holtzapple (2000) demonstrated that lime pretreatment significantly reduces lignin content and completely removes acetyl groups from hemicellulose. Physical pretreatments (e.g., ball milling) are highly effective at lowering cellulose crystallinity (Puri, 1984). Furthermore, combining lime pretreatment with mechanical pretreatment dramatically improves enzymatic digestibility (Falls and Holtzapple, 2011).

The purpose of this work is to generate highly digestible forage sorghum, a potential source of lignocellulose, to supplement or replace corn grain as

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ruminant animal feed. To accomplish this, a combination of oxidative lime pretreatment (OLP) and mechanical pretreatment (ball milling) were employed to render the biomass more digestible. To determine the nutritive value of the generated feed, composition and in vitro digestibility were determined.

## MATERIALS AND METHODS

### *Biomass Feedstocks*

Forage sorghum was harvested locally in College Station, Texas. The entire forage sorghum plant was dried to uniform moisture content (< 10%) before being ground to approximately 1 cm using a commercially available chipper. Dairy One Forage Laboratory (Ithaca, NY) performed the compositional analysis (Table 1). Samples were analyzed for dry matter (DM; AOAC, 2000a), ash (AOAC, 2000b), crude protein (CP; AOAC, 2005a), lignin, acid detergent fiber (ADF), neutral detergent fiber (NDF; ANKOM A200 Filter Bag Technique with F57 bag), crude fat (AOAC, 2005b), and non-fiber carbohydrates (NFC, AOAC, 1990). Two materials were used as controls: cracked corn grain and alfalfa. Cumberland Valley Analytical Services, Inc. (Hagerstown, MD) performed compositional analysis on these control materials (Table 1) using the same methods described above.

### *Short-term Oxidative Lime Pretreatment*

The entire forage sorghum plant was pretreated using short-term OLP as described by Sierra et al. (2009) and

Falls et al. (2011). Sorghum (8 g, dry basis), excess lime (5 g), and water (120 mL) were mixed in a 304 stainless steel pipe reactor (3.8-cm I.D., 12.7-cm long). The reactor [Fig. 1(a)] was sealed and connected to a swing arm located in a temperature-controlled oven. Pure oxygen ( $6.89 \times 10^5$  Pa) was provided to the reactor through a flexible hose attached directly to an oxygen cylinder (Praxair Distribution, Inc., Bryan, TX). The reaction was performed at 180°C for 2 h. Once complete, the reaction was quenched by placing the reactor in an ice-water bath. The reactor was slowly opened to relieve pressure, and the reactor contents were transferred to a 1-L plastic centrifuge bottle (Thermo Scientific Nalgene, Rochester, NY). The pretreated slurry was neutralized to pH 4.0 using 5-N HCl. The slurry was then vacuum filtered to isolate the pretreated solids. To wash out any residual lime, the pretreated solids were washed with distilled water a minimum of 3 times, until the pH of the collected wash was equal to that of fresh distilled water. The pretreated sorghum was air dried in metal pans. (Medical Action, Mechanicsville, VA) To prevent microbial growth in wet spots, the biomass was stirred at least once every 24 h. A portion of the short-term OLP sorghum was ball milled.

### *2.2.2 Long-term Oxidative Lime Pretreatment*

Additionally, forage sorghum was pretreated using long-term OLP. This pretreatment was performed in a series of 15 packed-bed reactors [Fig. 1(b)] made of PVC pipe (2.5 cm Sch. 40, 48.3 cm long). To maintain the desired reaction temperature (55°C), the reactors were jacketed with a larger diameter PVC pipe (5.1 cm

**Table 1.** Compositional analysis of raw feedstocks and treated forage sorghum

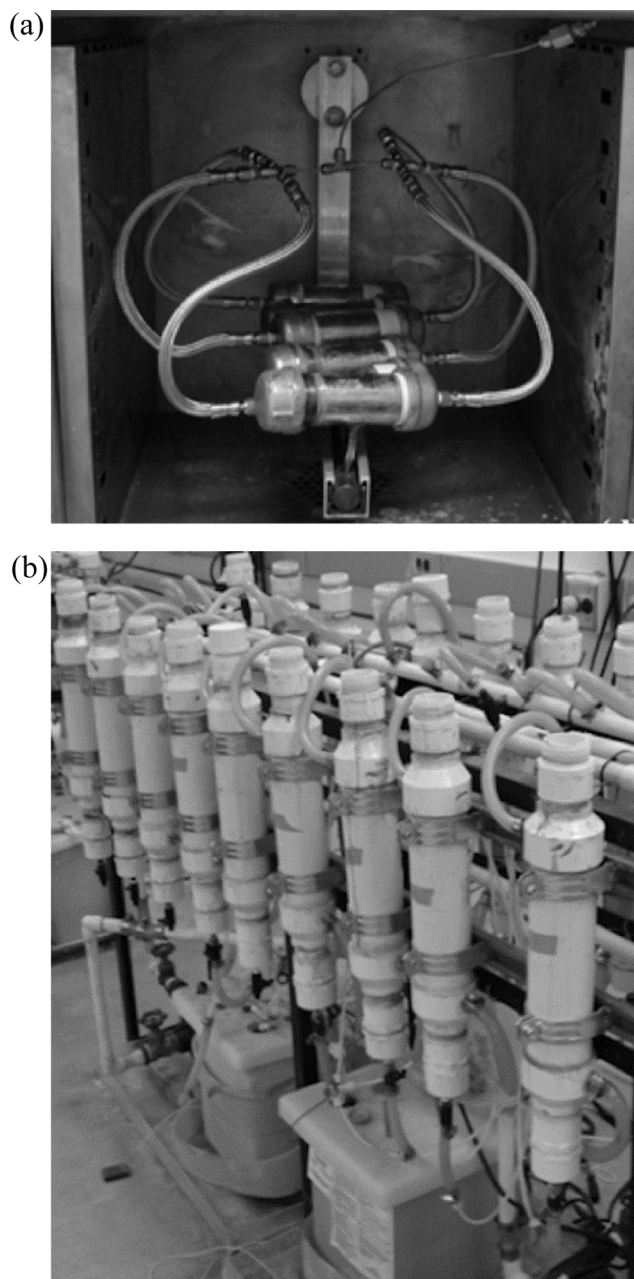
Sample	Forage sorghum					Corn grain	Alfalfa
	Raw	Short-term OLP <sup>1</sup>	10% Long-term OLP	20% Long-term OLP	30% Long-term OLP		
Moisture, %	8.6	—	—	—	—	14.2	7.4
Ash, % DM <sup>2</sup>	12.9	5.7	11.8	26.6	32.2	1.3	9.4
CP, % OM <sup>3</sup>	15.3	3.1	9.3	9.7	5.5	8.6	16.6
ADF, %OM	56.5	80.5	74.9	74.9	80.7	4.8	39.3
NDF, % OM	72.8	90.1	88.1	83.3	89.5	11.4	49.2
NFC <sup>4</sup> , % OM	15.6	10.8	7.6	9.3	5.9	76.6	34.1
Lignin, % OM	7.7	1.5	12.1	11.9	5.2	2.4	9.6
Fat, % OM	1.7	0.6	0.8	0.8	0.7	4.0	2.1
Ca, % DM	0.5	1.2	3.7	10.1	10.5	0.0	1.6
P, % DM	0.5	0.1	0.2	0.2	0.2	0.3	0.2
Mg, % DM	0.4	0.1	0.1	0.2	0.2	0.1	0.7
K, % DM	2.9	0.1	0.1	0.1	0.0	0.4	2.3
Na, % DM	0.0	0.0	0.0	0.0	0.0	0.0	0.1

<sup>1</sup>OLP = oxidative lime pretreatment.

<sup>2</sup>DM = dry matter.

<sup>3</sup>Organic matter (OM) = nutrient detergent fiber (NDF) + NFC + Fat + crude protein (CP).

<sup>4</sup>NFC = non-fiber carbohydrates.



**Figure 1.** (a) Stainless steel pipe reactor used for short-term oxidative lime pretreatment of forage sorghum; (b) Long-term-lime pretreatment reactor.

Sch. 40, 43.2 cm long) and water was pumped from a temperature-controlled tank. The oxidant employed was compressed air, which was scrubbed of carbon dioxide, preheated to the reaction temperature, and then bubbled through an inlet located at the bottom of each reactor.

Three different lime loadings were studied: 0.1 g lime/g dry sorghum (Reactors 1 through 5), 0.2 g lime/g dry sorghum (Reactors 6 through 10), and 0.3 g lime/g dry sorghum (Reactors 11 through 15). For each reactor, sorghum (80 g, dry basis) and the appropriate amount of lime were thoroughly mixed in a stainless steel tray before being loaded. Water was then added to each reactor until the biomass/lime mixture was completely submerged. The water level was

checked daily and additional water was added if necessary. The initial pH was 12.0, and the pretreatment was considered complete when the pH decreased to approximately 7.0. This resulted in a reaction time of 8 d for the 10% lime loading, 22 d for the 20% lime loading, and 34 d for the 30% lime loading. Once complete, the pretreated material was removed from the reactor and thoroughly washed with distilled water to remove any unreacted lime or lignin residue. The material was then air dried to uniform moisture content of less than 10%. Half of each pretreated sample was then ball milled.

### **Ball Milling**

Ball milling was used to decrystallize forage sorghum samples. Raw or pretreated whole-plant forage sorghum (6 dry g) was dried to less than 10% moisture and then transferred into a 300-mL porcelain jar. The porcelain jar was then loaded with 1.0-cm-diameter zirconia grinding medium (ER Advanced Ceramics, Inc., East Palestine, OH). The grinding medium was loaded to fill 50% of the jar volume (approximately 258 g). The jars were sealed and placed on rollers rotating at 68 rpm for 3 d. Metal sieve trays and a shaking apparatus were used to recover the ball-milled forage sorghum from the grinding media.

### **Total Digestible Nutrients**

Total digestible nutrients (TDN) were used to estimate the energy value of each prepared sample as animal feed. The TDN values were calculated using Eq. 1 (Tedeschi et al., 2009).

$$\text{TDN} = 0.98 \times [100 - (\text{NDF} - \text{NDIN}) - \text{CP} - \text{EE} - \text{Ash}] + \text{dCP} + \text{dEE} + \text{dNDF} - 7 \quad [1]$$

$$\text{dCP} = [1 - 0.004 \times (\{\text{ADIN} \times \text{CP}\} / 100)] \times \text{CP} \quad [2]$$

$$\text{dEE} = 2.25 \times (\text{EE} - 1) \quad [3]$$

$$\text{dNDF} = \text{NDFD}_{48} \times (\text{NDF} - \text{NDIN}) \quad [4]$$

where NDIN = neutral detergent insoluble nitrogen, ADIN = acid detergent insoluble nitrogen, EE = ether extract, dEE = digestible EE, dCP = digestible CP, and dNDF = ruminal and intestinal digestible NDF. All values, except ADIN, are expressed as percentages of the DM. The 48-h neutral detergent fiber

digestibilities (NDFD<sub>48</sub>) in Eq. 4 were calculated by Dairy One Forage Laboratory and Cumberland Valley Analytical Services, Inc.

### *In Vitro Neutral Detergent Fiber Digestibility*

Dairy One Forage Laboratory analyzed sorghum for 24- and 48-h in vitro neutral detergent fiber digestibility (NDFD) using the Ankom Daisy II Filter Bag Technique (ANKOM Technology, Macedon, NY). Rumen fluid was collected from a total-mixed-ration fed, high-producing lactating cow. The sorghum samples were incubated in a buffer (Goering and Van Soest, 1970) and rumen fluid mixture for 24 and 48 h under anaerobic conditions at 39°C. The remaining residue was used to determine NDFD.

### *Experimental Design*

To study the effect of OLP and ball milling, 10 sorghum samples were analyzed: (1) untreated; (2) ball milled; (3) short-term OLP; (4) short-term OLP + ball milled; (5 through 7) 10, 20, and 30% long-term OLP; and (8 through 10) 10, 20, 30% long-term OLP + ball milled. Dairy One Forage Laboratory analyzed these samples for composition and 24- and 48-h NDFD. Additionally, 2 control samples (i.e., corn grain and alfalfa) were analyzed by Cumberland Valley Analytical Services, Inc.

### *Model Development and Assessment*

A second-order polynomial ( $y = ax^2 + bx + c$ ) was used to represent the relationship between  $y$  [TDN, NDFD, or in vitro true digestibility (IVTD)] and  $x$  (lime loading in long-term OLP). The model parameters ( $a$ ,  $b$ , and  $c$ ) were obtained by minimizing the residual sum of squares with the Levenberg-Marquardt technique in Auto2Fit 5.5. Using this software, the root mean square error (RMSE) for each dataset was calculated (Eq. [5]).

$$\text{RMSE} = \sqrt{\frac{1}{n} \sum_{i=1}^n (y_i - \hat{y}_i)^2} \quad [5]$$

where  $n$  = number of experiment data,  $y_i$  = experiment value,  $\hat{y}_i$  = estimated value.

To determine if three adjustable model parameters ( $a$ ,  $b$ , and  $c$ ) are required when perhaps two ( $a$  and  $b$ ) would suffice, model assessment was performed by the  $F$ -test. This test is based on the regression sum of squares and the residual sum of squares (Eq. [6]).

$$F_c = \frac{\sum_{i=1}^n \frac{\hat{y}_i^2}{p}}{\sum_{i=1}^n \frac{(y_i - \hat{y}_i)^2}{n-p}} \quad [6]$$

where  $\hat{y}_i$  = estimated value of dependent value,  $y_i$  = measured data,  $p$  = number of parameters in the model, and  $n$  = number of experiments. As a reference,  $F(p, n-p; 1-\alpha)$  is the tabulated  $F$ -distribution with  $p$  and  $n-p$  degrees of freedom for a given confidence ( $\alpha$ ). If  $F_c$  is larger than  $F(p, n-p; 1-\alpha)$ , the regression is considered to be meaningful (Froment et al., 1990). For each model discussed in this article, the obtained  $F_c$  was higher than  $F(p, n-p; 90\%)$ ; therefore, the three-parameter model is valid.

## RESULTS AND DISCUSSION

The purpose of this research is to increase the ruminant digestibility of forage sorghum using OLP and ball milling. Oxidative lime pretreatment reduces lignin content and removes acetyl groups from hemicellulose (Chang et al., 1998; Rabelo et al., 2009; Saha and Cotta, 2008; Wyman et al., 2009), whereas ball milling decrystallizes cellulose (Bertran and Dale, 1985).

### *Compositional Analysis*

Table 1 shows the compositional analysis. Because some samples were not fully washed of all unreacted lime, the ash content was high. To compensate for this, all discussions will be on an organic (ash-free) basis. Furthermore, the following discussion will focus on 3 key components: NDF, NFC, and CP.

Neutral detergent fiber is comprised of the structural components of the plant cell wall: cellulose, hemicellulose, and lignin. Typically, it is one of the least digestible components of plant forage. Highly digestible ruminant feeds generally have very little NDF content. For example, corn grain generally has about 10% NDF (NRC, 2000). In contrast, the raw forage sorghum had 72.8% NDF. After OLP, NDF consistently increased for all conditions. Short-term OLP increased NDF content to 90.1%. The 10, 20, and 30% long-term OLP increased NDF content to 88.1, 83.3, and 89.5%, respectively. Beneficially, lime pretreatment removes lignin from the NDF; thus, an increase in NDF does not necessarily result in a lower digestibility feed. Unfortunately, lime pretreatment solubilizes some valuable components (e.g., CP and NFC). Although these valuable components could potentially be recovered from the pretreatment liquor, this option was not investigated in this study.

Ball milling generated small particles that introduced significant error in compositional analysis procedures that use filtering and gravimetric approaches. For all ball-milled samples, this resulted in inconsistent and highly unlikely values for both NDF and NFC; however, because ball milling is simply a mechanical process, the chemical composition of the feed should not be affected. Based on this reasoning, the chemical composition of ball-milled biomass was assumed to be identical to the un-ball-milled biomass.

Non-fiber carbohydrates are important because of their high inherent digestibility. The NFC of raw sorghum was quite low (15.6%) when compared to corn grain (76.6%), and even alfalfa (34.1%). Short-term OLP decreased NFC to 10.8%. The long-term OLP showed similar decreases with the 10, 20, and 30% lime loadings resulting in NFC content of 7.6, 9.3, and 5.9%, respectively. Traditionally, NFC is a highly digestive component, so any reduction in NFC is considered a negative consequence of pretreatment. For pretreatment to be worthwhile, significant gains must be shown elsewhere.

Protein is an important component of any diet, and raw sorghum had a CP content of 15.3%. Unfortunately, protein degradation is another negative aspect of the OLP process. The high temperature used in short-term OLP resulted in the harshest protein degradation, with a CP content of only 3.1%. The 10, 20, and 30% long-term lime pretreatments had CP contents of 9.3, 9.7, and 5.5%, respectively.

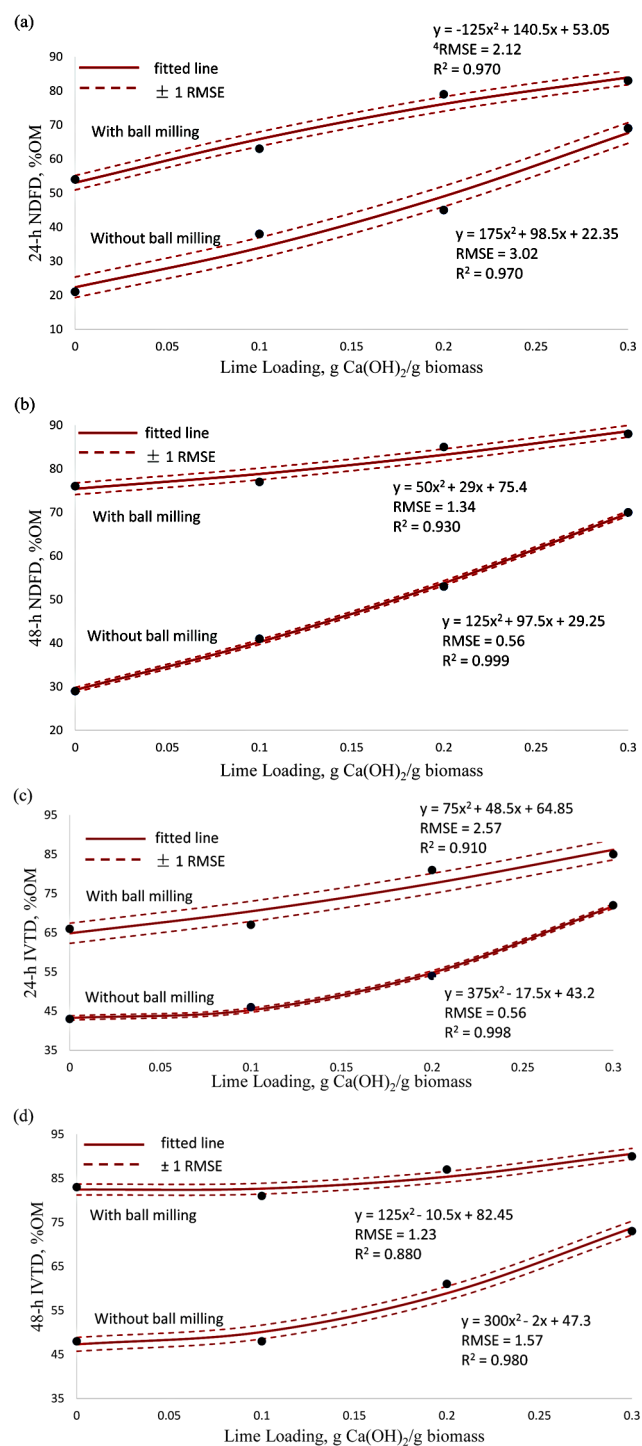
Mineral composition was mostly unaffected by OLP (Table 1). Oxidative lime pretreatment increased calcium significantly; however, this was primarily caused by unreacted lime resulting from not thoroughly washing the sorghum after pretreatment. Oxidative lime pretreatment did remove small amounts of phosphorous and magnesium, typically about 0.2% on a DM basis. Neither raw nor treated forage sorghum had measurable quantities of sodium.

In terms of compositional changes, OLP of sorghum increases NDF and decreases NFC and CP, all of which would traditionally be considered negative. To better understand the effect of OLP on lignocellulosic animal feed, it is necessary to study the digestibility of each component, particularly NDF.

### Neutral Detergent Fiber Digestibility and In Vitro True Digestibility

Neutral detergent fiber digestibility and IVTD of ball-milled materials were calculated based on the composition of their corresponding un-ball-milled materials because the chemical composition of the feed is assumed to be unchanged after ball milling.

Figures 2(a) and 2(b) show the effect of long-term OLP and ball milling on 24- and 48-h NDFD. Raw forage sorghum was used as the control, and had a 24-h NDFD (g NDF digested/100 g NDF incubated) of 21 and 48-h NDFD of 29. Lime pretreatment and ball milling increased both 24- and 48-h NDFD. Neutral detergent fiber digestibility of long-term OLP improved with



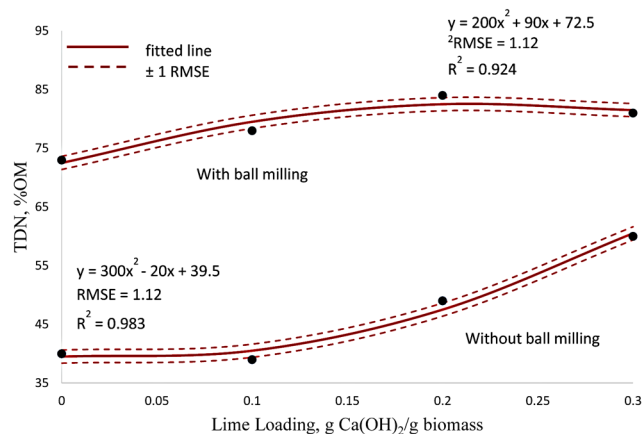
**Figure 2.** Effect of long-term <sup>1</sup>OLP and ball mill on (a) 24-h <sup>2</sup>NDFD, (b) 48-h NDFD, (c) 24-h <sup>3</sup>IVTD, (d) 48-h IVTD for forage sorghum. <sup>1</sup>OLP = oxidative lime pretreatment. <sup>2</sup>NDFD = neutral detergent fiber digestibility. <sup>3</sup>IVTD = in vitro true digestibility. <sup>4</sup>RMSE = root mean square error.

increased lime loading. Ball milling further increased NDFD of each long-term sample. For 48-h NDFD, 10, 20, and 30% long-term OLP + ball mill have values of 77, 85, and 88, respectively. Short-term OLP + ball mill (not shown) was the most digestible, digesting 89% of NDFD in both 24 h and 48 h.

In vitro true digestibility was also determined for forage sorghum sample [Fig. 2(c) and 2(d)]. All results discussed are 48-h IVTD reported on a percent organic basis. Raw forage sorghum had an IVTD of 48, which ball milling increased to 83. Short-term OLP and short-term OLP + ball mill had IVTD values of 72 and 89, respectively. In vitro true digestibility of long-term OLP improved with increased lime loading, with 10, 20, and 30% long-term OLP having values of 48, 61, and 73, respectively. Ball milling further increased IVTD of each long-term samples, with 10, 20, and 30% long-term OLP + ball mill having values of 81, 87, and 90, respectively.

### Total Digestible Nutrients

Figure 3 shows the effect of long-term OLP and ball mill on TDN. Total digestible nutrients was calculated using Eq. 1 based on the compositional analysis and 48-h NDFD results. All TDN values are reported as g nutrients digested/100 g organic matter fed (TDNom). As with NDFD, raw forage sorghum was used as the control and had a TDNom of 40. In terms of OLP, short-term OLP was the most successful, having TDNom values of 64. Long-term OLP improved with increased lime loading, with 10, 20, and 30% long-term OLP having TDNom values of 39, 49, and 60, respectively. Ball-milling improved TDNom in every case, with short-term OLP + ball mill (84) resulting in the highest TDNom values observed.



**Figure 3.** Effect of long-term <sup>1</sup>OLP and ball mill on total digestible nutrients (TDN) for forage sorghum. <sup>1</sup>OLP = oxidative lime pretreatment. <sup>2</sup>RMSE = root mean square error.

### Conclusions

With forage sorghum, OLP improved the NDF digestibility. Furthermore, adding ball milling improved the digestibility even more. This result is consistent with previous studies that have shown that adding ball milling to OLP significantly improves enzymatic digestibility, which should correlate well with ruminant digestibility (Bals et al., 2010; Falls and Holtzaple, 2011). Unfortunately, even if the rumen digestibilities are high, this treatment process has no practical use for animal feed because the fine particles can readily escape from the rumen before they are digested. Furthermore, ball milling is very expensive.

Although not practical, these data elucidate the value of combining chemical and mechanical pretreatments. Lessons from the results of this research can be summarized as follows: (1) use feedstock with low protein content, which prevents its loss in OLP; (2) extensively wash the biomass to remove ash from OLP; and (3) select a mechanical pretreatment that maintains fiber integrity so it is retained in the rumen until digested. These lessons are applied in Part 2, which investigates shock pretreatment, a new mechanical pretreatment that promises to be scalable and economical.

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