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Pre-treatment of sugarcane bagasse with a combination of sodium hydroxide and lime for improving the ruminal degradability: optimization of process parameters using response surface methodology

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A three-factor, three-level, face-centred cubic design was adopted to investigate the effect of three pre-treatment parameters, namely sodium hydroxide-to-dry biomass ratio [0.00, 0.05, and 0.10 (w/w)], lime-to-dry biomass ratio [0.00, 0.05, and 0.10 (w/w)] and residence reaction time (24, 96, and 168 h), at moderate temperature (21°C) on 24-h cumulative gas production (GP₂₄) and delignification, as the response variables. Under the optimal pre-treatment conditions of NaOH-to-dry biomass ratio of 0.09 (w/w), lime-to-dry biomass ratio of 0.09 (w/w) and residence time of 160.3 h, the model predicted 151.6 mL gas/g organic matter (OM) versus the experimental value of 147.2 mL gas/g OM. Under the optimal conditions of NaOH-to-dry biomass ratio of 0.10 (w/w), lime-to-dry biomass ratio of 0.08 (w/w) and residence time of 156.8 h, sugarcane bagasse (SCB) was maximally delignified by 41.2%, whereas the model predicted 43.6% delignification. Under the optimal conditions determined for GP₂₄, the neutral detergent fibre degradability of the pre-treated biomass after 24 and 48 h was 37.2 and 56.3%, respectively, a 1.90-fold and 1.58-fold over the untreated biomass, respectively. Overall, it was found that combined alkali treatment at optimal conditions effectively enhanced the ruminal degradability of SCB.

Keywords: *in vitro* degradability; lime; pre-treatment; sodium hydroxide; sugarcane bagasse

1. Introduction

It is projected that with an increasing world population (a 65% increase by ~2050), the global demand for food to feed the future generation will grow constantly (Wallace 2000). Ruminal microbial communities specialize in degrading the cellulosic biomass (Hess et al. 2011), enabling the ruminant animal to utilize lignocellulose, the most plentiful renewable biomass in nature (Alvira et al. 2010). Sugarcane bagasse (SCB), a crushed fibrous residue of sugarcane stalks remaining after juice extraction (Ramli et al. 2005), is an abundant lignocellulosic biomass with an annual production of approximately 54 million tons throughout the world (Samariha & Khakifirooz 2011).

Effective utilization of SCB as animal feedstuff is advantageous first for lessening the food crisis by presenting an inexpensive, readily available and abundant alternative feed, and second for alleviating the waste disposal problems facing the sugar industry (Ramli et al. 2005). Because of its high lignin content, SCB cannot efficiently be digested in the rumen (Okano et al. 2007);

therefore, an effective pre-treatment is needed to increase the digestibility of SCB for ruminants.

Alkaline pre-treatment is a highly effective pre-treatment which makes lignocellulose swollen and porous (Hendriks & Zeeman 2009), mainly through hemicellulose solubilization, and solubilization and/or redistribution of lignin (Lynd et al. 2002). These changes enhance the enzymatic hydrolysis during cellulosic-ethanol production process, which is highly correlated with improved ruminal degradability, because many fibrolytic enzymes of ruminal microorganisms are similar to the commercial enzyme cocktails, which are used during the enzymatic hydrolysis in lignocellulosic-ethanol production process (Anderson et al. 2010).

Although high-temperature alkali pre-treatment is fast and effective (Rezende et al. 2011; Ahmadi et al. 2013), but it is a high-energy demanding process, requiring heat- and corrosion-resistant equipment. Moreover, harsh pre-treatment conditions may lead to reactions that form carbon-carbon bonds between the lignin subunits; this can limit delignification and, subsequently

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decrease the enzymatic hydrolysis of carbohydrates (Pan et al. 2004). Therefore, pre-treatment at moderate temperatures may hold a promise for a low-cost and efficient method for conversion of SCB into a more digestible ruminant feed.

Lime [$\text{Ca}(\text{OH})_2$] pre-treatment at mild temperatures requires a long period of time to considerably enhance the digestibility of lignocellulose (Kim & Holtzapple 2005). Effective pre-treatment with NaOH can be achieved at faster rates using moderate temperatures (Wu et al. 2011), but is more costly [market price of \$450 and \$89.8 per ton for NaOH and lime, respectively (Kumar & Murthy 2011; Tao et al. 2011)]. However, by mixing NaOH and lime loadings, it may be possible to achieve a less expensive and at the same time an effective mixed alkaline pre-treatment at moderate temperatures (Xu & Cheng 2011).

Optimization of pre-treatment conditions is a key step in development of an efficient and cost-effective pre-treatment (Karunanithy & Muthukumarappan 2011). Response surface methodology (RSM), with successful application to lignocellulose pre-treatment (Velmurugan & Muthukumar 2012; Rawat et al. 2013), is an effective optimization tool with which many operating variables along with their interactive effects on a desired response and/or responses can be identified with few observations (Karunanithy & Muthukumarappan 2011). In comparison with conventional methods such as classical one-at-a-time, RSM offers a time-saving, cost-effective approach because many variables are studied at the same time with a low number of experimental trials (Deepak et al. 2008).

The present study was aimed at determining the optimal pre-treatment conditions for maximum GP_{24} and delignification, as response variables, using RSM and proposing mathematical equations to predict these responses. Amongst the various incubation times, GP_{24} is an important incubation time with an *in vivo* correlation, which is widely used to predict metabolizable energy of feed (Menke & Steingass 1988). Therefore, GP_{24} was chosen as a response for evaluating the pre-treatment efficiency. Because of the very high positive correlation of ethanol yield during simultaneous saccharification with fermentation and GP_{24} (Weimer et al. 2005), the data of this experiment might provide a valuable predictor of ethanol production from the pre-treated substrates.

2. Material and methods

2.1. Feedstock preparation

Dried unmilled SCB was obtained from Khuzestan Sugarcane Factory in southwestern Iran, and its particle size distribution was determined by using the Penn State

Particle Separator (Heinrichs & Kononoff 1996), as follows: >1.91 cm (9.1% of particles), $1.91\text{--}0.79$ cm (37.8%), $0.79\text{--}0.18$ cm (37.7%) and <0.18 cm (15.4%).

2.2. Pre-treatment procedure

Alkaline solutions were prepared by dissolving alkali reagents (lime or NaOH) with 300 mL distilled water. A 30-g sample of SCB [dry matter (DM) basis] was mixed thoroughly with the solution at a ratio of 1-g solid to 10 mL of solution in a 500-mL bottle. Because carbon dioxide in headspace may react with lime, the bottle was purged with a stream of N_2 for 60 s, after which the bottle was tightly screw-capped and stirred at 500 rpm for 10 min. Because lime is poorly soluble in water [about 1.5–1.6% at ambient conditions (Tao et al. 2011)], it requires shaking during the pre-treatment, which was provided by horizontal shaking of bottles inside a shaking water-bath (Mettmert, Model WNE 10, Schwabach, Germany) set at 30 back-and-forth movements per minute. Each pre-treatment run was replicated three times.

2.3. Determination of insoluble solid contents

At the end of each pre-treatment time, the slurry was harvested, pH was adjusted to around 7.0 by adding 1-N HCl and then vacuum-filtered (Whatman No. 41). The remaining solid was washed with distilled water and then filtered. The washing-filtration process was repeated three times or until the filtrate became colourless. The solid mass was dried in an air-forced oven for 48 h at 45°C . The solid recovery rate was determined gravimetrically (Kim 2004). A portion of each pre-treated sample was then used to determine chemical composition and *in vitro* gas production potential.

2.4. Compositional analysis

The oven-dried pre-treated or untreated bagasse samples were milled to pass through a 1-mm diameter screen using a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA). DM (method ID 930.15), crude protein (CP, method ID 984.13) and ash (method ID 942.05) were determined according to AOAC (2000). Ash-excluded neutral detergent fibre (NDF), without heat stable α -amylase, and acid detergent fibre were determined by an ANKOM²⁰⁰ Fiber Analyzer (Ankom Technology Corp., Fairport, NY, USA) according to Van Soest et al. (1991). Acid detergent lignin was measured using an ANKOM^{200/220} Daisy^{II} Incubator (ANKOM Technology, Fairport, NY, USA) with 72% (w/w) sulfuric acid according to Ankom protocol.

Delignification (D) was defined according to the following equation:

$$D = 1 - \left[\frac{L \times Y_T}{L_0} \right] \quad (1)$$

where L and L_0 are lignin content (%) in pre-treated and untreated biomass, respectively, and Y_T is the pre-treatment yield of the total insoluble solids determined after the pre-treatment (Kim & Holtzaple 2006).

2.5. *In vitro* gas production assay

Prior to morning feeding, rumen fluid was harvested from two non-lactating fistulated Holstein cows fed a diet of wheat straw (30%), concentrate mix (40%) containing mineral/vitamin supplements and alfalfa hay (30%). Details for preparation of rumen fluid and artificial saliva were as described earlier (Ahmadi et al. 2013). Gas volume in each volume-calibrated serum bottle, sealed with flanged butyl rubber stopper and aluminium crimp seal, was measured using a water displacement apparatus (Fedorak & Hrudey 1983) after 2, 4, 6, 8, 12, 16, 24, 48 and 72 h of incubation. Net gas production of each bottle at each incubation time was calculated after subtraction of the average gas production from four blank bottles (containing buffered rumen fluid but no substrate). Gas data were fitted using a first-order exponential model (Ørskov & McDonald 1979). Average fermentation rate (AFR) was calculated according to the equation proposed by Hervás et al. (2005). *In vitro* dry matter degradability (IVDMD) was determined gravimetrically by centrifuging the contents of each bottle (after 72 h of incubation) at $3000 \times g$ for 10 min, discarding the supernatant and drying the precipitate at 65°C to a constant weight (Mellenberger et al. 1970). Data on gas production were recorded from two separate runs, with three replicate bottles per treatment for each run.

2.6. Determination of NDF degradability

Degradability of NDF (at 24 and 48 h) for both untreated and optimum pre-treated SCB was determined by filling F-57 ANKOM bags (25- μm pore size) with approximately 500-mg substrate, transferring the bags to a loose mesh sack (2-mm pore size) and then incubating in the rumen. The bags were removed from the rumen after 24 or 48 h, hand-washed with cold water until the rinsing water became colourless and then dried at 60°C for 48 h (Ghasemi, Ghorbani et al. 2014). Ruminant degradability of NDF was determined gravimetrically by an ANKOM²⁰⁰ Fiber Analyzer (Van Soest et al. 1991).

2.7. Experimental design

The influence of three independent variables, namely sodium hydroxide-to-dry biomass ratio (w/w), lime-to-dry biomass ratio (w/w) and residence reaction time (h), on GP₂₄ and delignification (as response variables), was determined using RSM. The range of each pre-treatment variable was determined based on economic feasibility and preliminary study, and each independent variable at three levels was coded according to: -1, 0 and +1 (Table 1). A three-factor, three-level, face-centred design with 20 experimental runs and 6 replicates at the centre points (Table 2) was developed using Design-Expert[®] software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA). The experimental data were fitted to following second-order polynomial model (Equation 2):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \varepsilon \quad (2)$$

where Y is the dependent variable (GP₂₄ or delignification); β_0 is the intercept coefficient; β_1 , β_2 and β_3 are linear coefficients; β_{11} , β_{22} and β_{33} are quadratic coefficients; β_{12} , β_{13} and β_{23} are interaction coefficients; A , B and C represent the sodium hydroxide-to-dry biomass ratio (w/w), lime-to-dry biomass (w/w) and pre-treatment time (h), respectively; and ε is the error term. The effects of variables and their interactive effects were determined by analysis of variance (ANOVA). The data were presented as means, and the level of significance was set at $P < 0.05$.

2.8. Data analysis and model verification

Using the predictive equations of RSM, the optimal alkaline pre-treatment conditions were determined, and output variables, GP₂₄ and delignification, were again measured under the proposed optimal conditions to verify the validity of the models. Under the optimal conditions determined for GP₂₄, SCB was pre-treated and subjected to some supplementary tests to gain a better insight into the influence of the pre-treatment on chemical composition and *in vitro* degradability of SCB. Data obtained from the supplementary tests were analysed using one-way ANOVA (SPSS software, Ver.

Table 1. Codes and levels of the independent variables used for optimization.

Independent variable	Unit	Symbol	Coded levels		
			-1	0	+1
NaOH-to-dry biomass ratio	w/w	A	0.00	0.05	0.10
Lime-to-dry biomass ratio	w/w	B	0.00	0.05	0.10
Residence time	h	C	24	96	168

Table 2. Experimental design of both coded and actual values for GP₂₄ and delignification, observed and predicted values.

Std. order	Run no.	Pre-treatment factors			GP ₂₄ (mL/g OM)			Delignification (%)		
		<i>A</i>	<i>B</i>	<i>C</i>	Obsd.	Pred.	Resl.	Obsd.	Pred.	Resl.
1	1	0.00 (−1)	0.00 (−1)	24 (−1)	70.6	70.1	0.5	3.8	4.1	−0.3
11	2	0.05 (0)	0.00 (−1)	96 (0)	110.5	102.7	7.8	21.7	19.4	2.3
13	3	0.05 (0)	0.05 (0)	24 (−1)	123.5	124.0	−0.5	17.8	22.3	−4.4
17	4	0.05 (0)	0.05 (0)	96 (0)	121.1	124.3	−3.2	25.7	25.4	0.4
10	5	0.10 (+1)	0.05 (0)	96 (0)	138.5	137.1	1.4	41.0	37.6	3.5
15	6	0.05 (0)	0.05 (0)	96 (0)	126.0	124.3	1.7	19.8	25.4	−5.6
18	7	0.05 (0)	0.05 (0)	96 (0)	119.8	124.3	−4.5	22.4	25.4	−2.9
14	8	0.05 (0)	0.05 (0)	168 (+1)	141.1	134.1	7.0	35.3	28.5	6.8
6	9	0.10 (+1)	0.00 (−1)	168 (+1)	127.8	128.3	−0.5	36.5	34.7	1.9
16	10	0.05 (0)	0.05 (0)	96 (0)	117.3	124.3	−7.0	26.4	25.4	1.0
8	11	0.10 (+1)	0.10 (+1)	168 (+1)	152.0	154.1	−2.1	43.6	46.7	−3.1
5	12	0.00 (−1)	0.00 (−1)	168 (+1)	67.8	73.1	−5.3	4.2	10.3	−6.1
7	13	0.00 (−1)	0.10 (+1)	168 (+1)	137.8	136.9	0.9	24.6	22.3	2.3
19	14	0.05 (0)	0.05 (0)	96 (0)	121.8	124.3	−2.5	21.2	25.4	−4.2
20	15	0.05 (0)	0.05 (0)	96 (0)	127.2	124.3	2.9	22.2	25.4	−3.2
12	16	0.05 (0)	0.10 (+1)	96 (0)	136.1	137.5	−1.4	32.0	31.4	0.6
4	17	0.10 (+1)	0.10 (+1)	24 (−1)	140.5	136.8	3.7	38.6	40.4	−1.8
3	18	0.00 (−1)	0.10 (+1)	24 (−1)	112.9	114.0	−1.1	20.1	16.1	4.0
2	19	0.10 (+1)	0.00 (−1)	24 (−1)	128.4	131.0	−2.6	32.7	28.4	4.2
9	20	0.00 (−1)	0.05 (0)	96 (0)	103.1	98.0	5.1	17.9	13.2	4.7

Note: Data represent six replicates (three replicate pre-treatments × two replicates per pre-treatment replicate).

A, *B* and *C* are NaOH-to-dry biomass ratio (w/w), lime-to-dry biomass ratio (w/w) and pre-treatment time (h), respectively.

Obsd., observed; Pred., predicted; Resl., residual; GP₂₄, cumulative gas volume produced during an incubation period of 24 h.

16.0.0, 2007, SPSS Inc., Chicago, IL, USA). The level of significance was set at $P < 0.05$ and mean comparison was performed by the Duncan's multiple range test.

2.9. Fourier transform infrared–attenuated total reflection (FT-IR–ATR) spectroscopy

FT-IR spectroscopic analysis was performed to detect changes in the functional groups of the pre-treated biomass. FT-IR spectra were recorded by averaging 16 scans per sample from 4000 to 600 cm^{−1} at a resolution of 4 cm^{−1} using FT-IR–ATR spectroscopy (Tensor 27 FT-IR spectrometer, Bruker, Billerica, MA, USA) at the central laboratory in Isfahan University of Technology, Isfahan, Iran.

3. Results

3.1. Effect of pre-treatment on GP₂₄

The results on GP₂₄ data (Table 3) showed that the model used to fit GP₂₄ data was highly significant ($P < 0.0001$), suggesting that the model adequately represented the relationship between the response and independent variables (Hossain et al. 2012). Determination coefficient (R^2) was determined to be 0.9661, indicating that some 96.6% of variability in the response variable could be explained by the model. The predicted R^2 (0.8255) was in close agreement with R^2_{adj} (0.9443; Table 3). The

precision and reliability of the experiments conducted for GP₂₄ were verified by a low value of 4.46% for the coefficient of variation (CV). The variation of the data around the fitted model is described by lack of fit; non-significant (P value = 0.1159) lack of fit indicated that the model is a good predictor of the response.

The second-order polynomial equation for GP₂₄ was as follows:

$$\begin{aligned}
 Y = & +73.34232 + 888.1741A + 575.79463B \\
 & - 0.15511C - 3803.36564AB - 0.38763AC \\
 & + 1.38366BC - 2704.46816A^2 \\
 & - 1701.86816B^2 + 0.000913505C^2
 \end{aligned} \quad (3)$$

in which, Y represents 24-h accumulative gas production [mL/g organic matter (OM)], and A , B and C are sodium hydroxide-to-dry biomass ratio (w/w), lime-to-dry biomass ratio (w/w) and pre-treatment time (h), respectively. The ANOVA revealed that lime loading and NaOH loading linearly affected ($P < 0.05$) GP₂₄. Interaction effects of lime loading and pre-treatment time ($P = 0.0262$), and also that of sodium hydroxide and lime loading on GP₂₄ were significant ($P = 0.0006$; Table 3).

The maximum GP₂₄ was determined to be 147.2 mL/g OM at NaOH-to-dry biomass ratio of 0.09 (w/w), lime-to-dry biomass ratio of 0.09 (w/w) and pre-treatment duration of 160.3 h. Under these conditions, the model

Table 3. ANOVA for response surface quadratic model for response variables, GP₂₄ and delignification.

Source	GP ₂₄ (mL g/OM)				Delignification (%)					
	DF	Sum of squares	Mean value	F value	P > F	DF	Sum of squares	Mean value	F value	P > F
Model	9	8324.37	924.93	31.63	<0.0001	3	1939.27	646.42	38.60	<0.0001
A	1	275.33	275.33	9.42	0.0119	1	1480.85	1480.85	88.42	<0.0001
B	1	163.63	163.63	5.60	0.0396	1	361.08	361.08	21.56	0.0003
C	1	68.43	68.43	2.34	0.1571	1	97.34	97.34	5.81	0.0283
AB	1	723.28	723.28	24.73	0.0006					
AC	1	15.58	15.58	0.53	0.4822					
BC	1	198.50	198.50	6.79	0.0262					
A ²	1	125.71	125.71	4.30	0.0649					
B ²	1	49.78	49.78	1.70	0.2212					
C ²	1	61.67	61.67	2.11	0.1771					
Residual Error	10	292.41	29.24			16	267.98	16.75		
Lack of Fit	5	222.20	44.44	3.16	0.1159	11	234.62	21.33	3.20	0.1049
Pure Error	5	70.21	14.04			5	33.36	6.67		
Total	19	8616.78				19	2207.25			

Note: A, B and C are NaOH-to-dry biomass ratio (w/w), lime-to-dry biomass ratio (w/w) and pre-treatment time (h), respectively.

For GP₂₄: CV = 4.46%; $R^2 = 0.9661$; Pred $R^2 = 0.7725$; Adj $R^2 = 0.9355$; Adeq precision = 21.956.

For delignification: CV = 16.12%; $R^2 = 0.8786$; Pred $R^2 = 0.7988$; Adj $R^2 = 0.8558$; Adeq precision = 23.274

Values of ' $P > F$ ' less than 0.05 indicate model terms are significant, revealing that for GP₂₄, B, C, AB and BC are significant model terms, whereas A, B, C are significant model terms for delignification.

DF, degree of freedom.

predicted GP₂₄ of 151.6 mL/g OM, which was in a close agreement with the experimental value. Figure 1a depicts the close agreement between the observed GP₂₄ (the response variable) values and those predicted from the model Equation (3).

3.2. Effect of pre-treatment on delignification

Delignification data on SCB pre-treated under different conditions ranged from 3.8% to 43.6% (Table 2). All three independent variables linearly influenced the degree of delignification ($P < 0.05$), but there were no significant interaction between these variables ($P > 0.05$). Under the optimal pre-treatment conditions (NaOH-to-dry biomass ratio of 0.10, lime-to-dry biomass ratio of 0.08 and

pre-treatment duration of 156.8 min), the model predicted 43.6% delignification. The degree of delignification obtained after using the proposed optimal conditions was 41.2%, a value very close to the predicted value. A high correlation was recorded between the predicted and experimental values (Figure 1b).

3.3. Changes in chemical composition of bagasse

Table 4 shows the compositional analysis of untreated, washed-only and optimally pre-treated SCB. A slight change was observed in chemical composition of washed-only bagasse (in comparison with untreated one), whereas the pre-treatment at optimum conditions caused the cellulose concentration to increase by 8.9%,

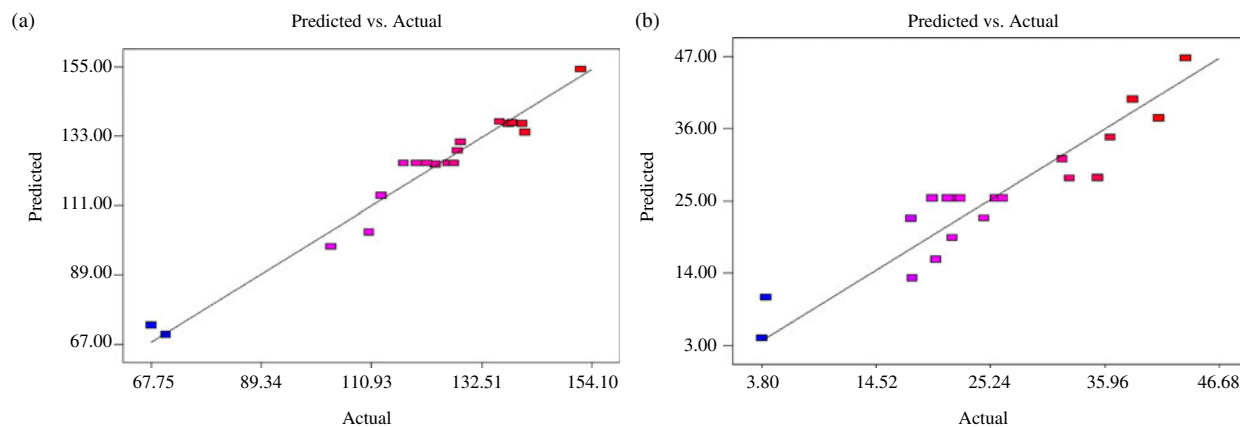


Figure 1. Experimental (actual) values versus predicted values for (a) GP₂₄ and (b) delignification.

Table 4. Chemical composition of SCB: untreated, washed-only and pre-treated under optimized conditions.

Items	Treatment			Statistics	
	Untreated	Washed-only	Pre-treated ¹	SEM	P value
Chemical composition (% of OM)					
Hemicellulose	31.2 ^a	31.9 ^a	21.8 ^b	2.78	<0.001
Cellulose	46.2 ^b	48.1 ^{ab}	50.3 ^a	2.31	0.042
Lignin	9.74 ^b	9.23 ^b	6.65 ^a	1.71	0.015
Crude protein	1.64 ^a	1.28 ^b	0.45 ^c	0.33	<0.001
Solid recovery (%)	—	94.9	85.3	2.04	<0.001

Note: Data represent the mean value of three replicate pre-treatments. Means within each row with different letters are significantly different ($P < 0.05$), Duncan's test.

¹Pre-treated under the optimal processing conditions of NaOH-to-dry biomass ratio of 0.09 (w/w), lime-to-dry biomass ratio of 0.09 (w/w) and residence time of 160.3 h.

SEM, standard error of the mean.

while it substantially solubilized that of hemicellulose by 30.4%. Protein degradation was a negative aspect of the pre-treatment, because it significantly decreased the crude protein content of bagasse (a reduction from 1.28% to 0.45%, for washed-only and optimum pre-treated biomass, respectively). Solid loss after washing alone was 5.1% compared with 14.7% for pre-treated materials under optimum processing conditions (Table 4).

3.4. Effect of pre-treatment on NDF degradability and gas production kinetics

To gain a better insight into the impact of the pre-treatment on ruminal degradability of SCB, ruminal degradability of NDF (NDFd) was determined. Untreated bagasse had a 24-h NDFd of 19.6% (19.6 g NDF digested/100 g NDF incubated) and 48-h NDFd of

35.7%, while the pre-treatment (optimum pre-treatment) improved both 24- and 48-h NDFd by 90.0% and 57.7%, respectively (Table 5).

Under the optimal pre-treatment conditions, gas production rate constant ($1/h$) increased significantly ($P = 0.001$) from 0.021 to 0.043 for the untreated and optimally pre-treated substrate, respectively (Table 5). The AFR of optimally pre-treated bagasse was 2.33 times that of untreated bagasse. Figure 2 illustrates the cumulative gas production profile (during an incubation period of 72 h) of untreated bagasse and bagasse pre-treated under the optimum conditions. The pre-treatment substantially improved the gas production profile of the treated bagasse; both untreated and treated bagasse had a similar profile of gas production during the first 8 h of fermentation, followed by an increasing rate of gas production for the pre-treated biomass, and about 72.8% of its total gas (202 mL/g OM) was produced within the first 24 h of fermentation.

Table 5. NDF degradability and fermentation kinetic of SCB: untreated and pre-treated under optimal conditions.

Items	Treatment		Statistics	
	Untreated	Pre-treated ^a	SEM	P value
Degradability (% of OM)				
24-h NDF	19.6	37.2	3.94	<0.001
48-h NDF	35.7	56.3	6.41	<0.001
IVDMD (% of DM)	41.8	62.7	4.92	<0.001
Fermentation kinetics				
c ($1/h$)	0.021	0.043	0.002	0.001
AFR [mL/(g OM·h)]	2.59	6.03	0.98	<0.001

Note: Means within each row with different letters are significantly different ($P < 0.05$), Duncan's test.

^aPre-treated under the optimal pre-treatment conditions of NaOH-to-dry biomass ratio of 0.09 (w/w), lime-to-dry biomass ratio of 0.09 (w/w) and residence time of 160.3 h.

IVDMD, *In vitro* dry matter degradability.

SEM, standard error of the mean; c , gas production rate constant; AFR, average fermentation rate.

3.5. FT-IR-ATR spectroscopic analysis

Chemical changes in the pre-treated SCB were characterized with FT-IR spectroscopy, which is a widely used technique for detecting the chemical changes in

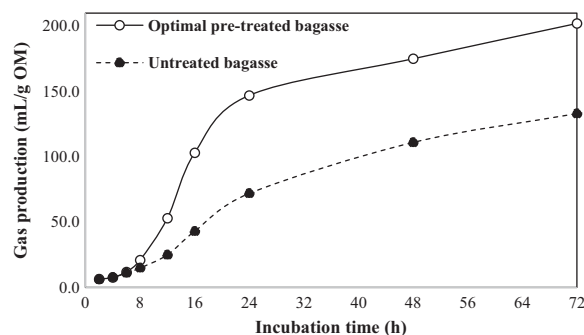


Figure 2. Fermentative gas production profile of SCB: untreated and pre-treated under optimized conditions.

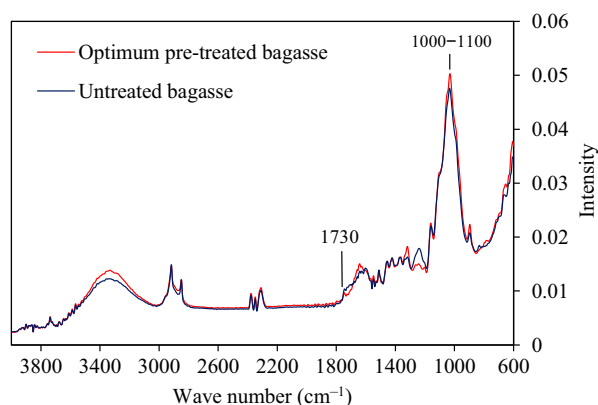


Figure 3. Chemical changes in SCB solids, as determined by FT-IR-ATR: untreated and pre-treated under optimized conditions.

lignocellulosic materials (Xu et al. 2007). Figure 3 illustrates the FT-IR spectra of untreated and optimally pre-treated SCB. The spectrum of optimally pre-treated SCB showed slight differences in some functional groups, as compared with the untreated SCB, suggesting that pre-treatment chemically transformed SCB. The saponifying impact of the mixed alkaline pre-treatment on SCB was verified by a 12% reduction in a band at 1730 cm^{-1} , which is attributed to saponification of the ester bonds, such as acetyl and uronic ester groups (Peng et al. 2012). An increase in absorptions peaks at $1000\text{--}1100\text{ cm}^{-1}$ in the spectrum of the pre-treated SCB solids demonstrated an increased concentration of cellulose in the pre-treated solid (Sun et al. 2008), as already shown by compositional analysis (Table 4).

4. Discussion

An ideal pre-treatment procedure aims at maximizing the substrate delignification while minimizing modification of polysaccharide, thereby enabling rapid digestion of pre-treated substrate by enhancing the accessibility of enzymes to polysaccharides (Ding et al. 2012). The effective delignifying power of both NaOH and lime has been demonstrated in several studies (Chen et al. 2007; Silverstein et al. 2007; Rezende et al. 2011; Shiroma et al. 2011; Xu & Cheng 2011). For example, Silverstein et al. (2007), comparing the delignification effect of four chemical reagents (NaOH, H_2SO_4 , H_2O_2 and ozone) on cotton stalks, showed that NaOH pre-treatment resulted in the highest lignin removal (65.6% at 2.0% NaOH, 90 min, 121°C). In another study, combination of sodium hydroxide [NaOH-to-raw biomass ratio of 0.1 (w/w) and lime-to-dry biomass ratio of 0.02 (w/w)] for 6 h at room temperature delignified switchgrass by 37.8% (Xu & Cheng 2011).

Of the three main components of SCB (i.e. hemicellulose, cellulose and lignin), the cellulosic fraction

was not significantly affected by pre-treatment with alkali, whereas hemicellulose was significantly solubilized. In a recent study, where corn stover was pre-treated with NaOH to increase its enzymatic hydrolysis, more than 95% of the cellulosic fraction was preserved (Chen et al. 2013); it was also shown that when NaOH loading increased from 0.04 to 0.1 g/g corn stover, degradation of hemicellulose (xylan) was increased by 20%. Rezende et al. (2011) pre-treated SCB with various loadings of NaOH to enhance its enzymatic digestibility; in general, compositional analysis of SCB pre-treated by various loading of NaOH showed that alkaline pre-treatment resulted in a significant increase in hemicellulose and lignin solubilization, whereas cellulosic fraction was well preserved and its concentration increased in retained solids. However, NaOH loadings above 2% did not further increase lignin and hemicellulose solubilization, and at these concentrations, cellulose loss began to increase. Overall, the results of that study indicated that NaOH pre-treatment with concentrations above 1% might not be effective (Rezende et al. 2011).

High recalcitrance and crystallinity of cellulose are the main factors reducing the sensitivity of cellulose with alkaline pre-treatment (Lai 1991; Gupta & Lee 2010). The reason why the pre-treatment significantly solubilized hemicellulosic fraction of bagasse results mainly from the fact that hemicellulose has an amorphous and branched structure, conferring little strength against alkali solubilization (Xu & Cheng 2011).

The high solid recovery after pre-treatment was expected, because sugarcane is extensively washed in sugar mill during sugar extraction process; for this reason, bagasse fibre becomes quite resistant to solubilization during the pre-treatment (Gandi et al. 1997). This is of great significance, because the economic viability of the pre-treatment process is highly dependent on high solid yield.

The enhancement in NDF degradability of the pre-treated SCB (both 24 and 48 h) (Table 5) is of paramount importance, since the main obstacle facing the development of a digestible lignocellulosic ruminant feed is overcoming its high NDF content, which has generally poor ruminal digestibility (Falls 2011).

The higher amount of gas produced after 8 h of fermentation in the pre-treated bagasse may be explained by an enhanced reactivity of NDF portion of pre-treated bagasse to ruminal fibrolytic microorganisms. The volume of gas produced between 6 and 24 h of incubation is largely attributable to NDF digestion in cattle fed at high production levels, while the volume produced between 6 and 72 h of fermentation would be attributable to the amount of NDF which will be digested in cattle fed at maintenance intake levels (Robinson & Getachew 2002). Thus according to Figure 3, the gas production profile of untreated and treated bagasse during an incubation

period of 72 h, it can be concluded that high-producing dairy cattle may not efficiently benefit the pre-treated biomass, because they have a fast ruminal passage rate (Gressley et al. 2011), which gives the ruminal micro-organisms a limited time for digestion of the pre-treated SCB. Hence, it would be more appropriate to consider its inclusion in the diet of cattle fed at the maintenance level, which have a slower ruminal passage rate.

Alkaline pre-treatment (using either lime or sodium hydroxide, or combination of them) for enhancing either ruminal digestibility or enzymatic digestibility in cellulosic-ethanol production process has been widely attempted (Haddad et al. 1998; Xu & Cheng 2011; Ahmadi et al. 2013; Chen et al. 2013). For example, combination of sodium hydroxide and lime to enhance the enzymatic hydrolysis in lignocellulosic-ethanol production process was reported by Xu and Cheng (2011) in which, at the recommended conditions [6 h, 21°C, 1.0% (w/w) NaOH, and 0.2% (w/w) Ca(OH)₂], the glucose and xylose yields of switchgrass reached 59.4% and 57.3% of their theoretical yields, respectively. Haddad et al. (1998) investigated the efficacy of treating wheat straw with various combinations of sodium hydroxide and lime using the spraying (dry) technique on the performance of lactating Holstein cattle and determined the optimum processing conditions as 3% NaOH + 3% Ca(OH)₂ (wt/wt, DM basis). Inclusion of the pre-treated wheat straw at 20% of diet of Holstein cows had minor impact on ruminal function and lactational performance (both short term and long term), in comparison with control diet which contained pre-bloom alfalfa hay. More recently, various combinations of sodium hydroxide and lime were used to improve ruminal degradability of date palm leaf; under the optimum processing conditions [71.4 h, 40°C, 0.6% (w/w) NaOH and 0.9% (w/w) Ca(OH)₂], gas production at 24 h was 2.46-fold over the untreated biomass (Rajae Rad et al. 2014). Enhanced ruminal degradability of the pre-treated SCB is most likely due to its lignin removal, since lignin, a highly oxygenated complex aromatic polymer which firmly complexes cellulose fibres (Lee et al. 2014), is an important impediment affecting the rate and extent of rumen digestion of fibre (Waghorn & McNabb 2003). By mitigating this barrier (Table 3), the accessibility of ruminal microorganisms to carbohydrate portion of the biomass may enhance, which consequently leads to an improved rumen degradability.

Supplementing lime with NaOH can be advantageous for animal feed purposes, because diets which are mainly composed of grains and mature forages are usually deficient in calcium (NRC 1984), thus mixing the calcium-enriched biomass with these diets can be beneficial for the ruminant animal (Gandi et al. 1997). After NaOH pre-treatment, high Na concentration may also exist in pre-treated biomass, but fortunately no

health problem or impaired performance has been reported in ruminants consuming NaOH-pre-treated biomass; excess sodium ingested is excreted in the urine leading to an increase in water intake and urinary volume (Ghasemi, Khorvash et al. 2014).

5. Conclusions

Pre-treatment using the optimal conditions significantly improved the ruminal degradability of SCB, which may be suitable for inclusion in the ruminant diet. However, *in vivo* studies are needed to evaluate the effect of dietary inclusion of the pre-treated substrate on the performance of ruminants. A comprehensive cost-benefit analysis is also needed to select the most cost-effective pre-treatment procedure.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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