Production of Fermentable Sugars and a High Protein Meal by Dilute Acid Hydrolysis of Soybean Meal at High Temperatures

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The objective was to produce fermentable sugars with low levels of fermentation inhibitors and a high-protein meal, with acceptable color, by treating defatted soybean meal with dilute sulphuric acid at temperatures between 105 and 135 °C and durations up to 45 min. The conditions that maximized the amount of protein were 120 °C, 0.5% H_2SO_4, and 45 min, which increased protein from 48.1% to 58.6% d.b. The highest amount of fermentable sugars (32.2% d.b.), without regard for the protein content, was for the treatment at 135 °C, 2% H_2SO_4, and 45 min; such treatment generated relatively low 5-HMF and furfural levels (0.0018 g/L and 0.32 g/L, respectively), and 0.87 g/L, of acetic acid. The treatment at 120 °C, 1.5% H_2SO_4, and 30 min had the best balance between a high concentration of fermentable sugars (21.3% d.b.) in the liquid fraction and crude protein (52.1% d.b.) in the solid fraction without a significant change in the original color of the solid fraction.

Keywords: Soybean meal; Hydrolysis; Dilute acid; Fermentable sugars; Hydroxymethylfurfural; Furfural

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INTRODUCTION

Soybean meal (SBM), a co-product of soybean oil extraction, provides high quality protein to the animal feed industry because of its protein content, amino acid composition, overall nutrient content, and high energy compared to other plant protein sources used as feedstock (Waldroup 2006). Protein content of commercial SBM can range between 44% and 49% (Kim *et al.* 2003). In addition, SBM contains sugars that can be extracted and used for fermentations while leaving as a co-product a high-protein meal that would provide more flexibility in formulation of animal rations and expand its use to other markets, such as aquaculture.

In previous work in our laboratory, Luján-Rhenals *et al.* (2014), showed that the protein content of soybean meal can be boosted with a treatment with dilute sulfuric acid. In that research, 48-percent-protein commercial SBM treated with 1.9 to 2.0% sulfuric acid for 7 to 16 h at 80 °C produced a protein-enriched solid fraction with 58% protein at the expense of reducing the soluble carbohydrates content and other soluble compounds. A byproduct of the process was a liquid fraction containing 21 grams of fermentable sugars per 100 g of untreated SBM that could be used for the production of the production of value-added products *via* fermentation.

The solid fraction obtained in our previous research still contains structural carbohydrates, especially cellulose and hemicelluloses, that could be further removed with

a different treatment and would result in increased protein content. Earlier reports by other authors using a variety of biomass sources including corn stover, switchgrass, sugarcane, and hardwoods indicated that hydrolysis with sulfuric acid at temperatures above 100 ºC led to higher reaction rates and more complete cellulose hydrolysis (Esteghlalian *et al.* 1997; McMillan 1994; Morjanoff and Gray 1987). Furthermore, these higher temperatures during acid hydrolysis degrade hemicelluloses, thus making cellulose more accessible to enzymes for further hydrolysis (Jacobsen and Wyman 2000). Therefore, it is valid to hypothesize that the treatment of SBM at temperatures above 100 ºC may increase the amount of sugars obtained in our previous research and still produce a protein fraction that could be used as an animal feed.

One problem that can result from these treatments is the degradation of sugars and the formation of undesirable by-products, such as 5-HMF, furfural, and acetic acid, all substances that can inhibit fermentation process (Lee *et al.* 1999a,b; Larsson *et al.* 2000; Buhner and Agblevor 2004). A second concern is the significant alteration of color, which has been observed when treating lignocellulosic materials with acid at high temperatures and the eventual degradation of protein as a result of non-enzymatic reactions of protein with reducing sugars.

The objective of this study was to determine the effect on protein content, color, production of inhibitors, and production of of fermentable sugars when treating SBM with less than 2% sulfuric acid at 105, 120, and 135 ºC for durations up to 45 min.

MATERIALS AND METHODS

Materials

Commercial SBM was obtained from a soybean processor in the state of Arkansas. The SBM was sieved with a U.S. standard sieve #10 and used without further treatment. Reagents used in this research were sulfuric acid (96.5%) from J. T. Baker (Phillipsburg, NJ, USA); 5-hydroxymethylfurfural (99%), and sodium hydroxide (99%) from Sigma Aldrich (St. Louis, MO, USA); and furfural (98%) from TCI (AMERICA-Sigma Aldrich). All the standards for sugar analysis were also obtained from Sigma-Aldrich.

Composition of Soybean Meal

Moisture content was analyzed in a conventional oven by drying 10 g SBM at 115 ºC until a constant weight between readings was obtained (about 24 h). The starch content was determined by the A.A.C.C. Enzyme Method 79-13 (A.A.C.C. 2000), the acid and neutral detergent-fiber (AD-fiber, and ND-fiber) by an ANKOM-200 (Macedon, NY, USA), and the ash content by method A.O.A.C. 923.03 (A.O.A.C. 1990a.).

Dilute Acid Hydrolysis

Hydrolysis experiments were conducted in duplicate in a Tuttnauer 2340E Steam Autoclave (Tuttnauer USA, Delran, NJ) using the preset program #1: "fast exhaust without drying." Samples were treated at 3 temperatures, 4 concentrations of sulfuric acid, and 3 durations arranged according to a split-plot experimental design with temperatures and times as the whole plot and the concentration of H_2SO_4 as the split-plot factor (Table 1).

Treatments were conducted in 500-mL PYREX® media storage bottles with screw caps. Fifty grams of SBM (dry weight) was placed in each flask along with 250 mL of

H_2SO_4 solution and the flask capped loosely to avoid pressure buildup. The flasks were placed in the autoclave and heated to the temperatures and durations indicated by the experimental design.

The reaction was stopped after the prescribed duration by cooling the flasks in an ice-water bath followed by the addition of NaOH pellets to raise the pH to the range 5.0 to 5.5. Transient heating and cooling was not considered in the analysis of the results.

After pH adjustments, centrifugation at 3900 x g for 35 min at 10 ºC separated the solids from the liquid. Supernatants were filtered through Whatman #4 filter paper (Whatman Plc., Maidstone, Kent, UK) and analyzed for fermentable sugars, 5-HMF, furfural, and acetic acid. Resulting sugars were expressed as grams of sugar per 100 grams of untreated meal (dry basis) and the inhibitors as grams per liter of the liquid fraction.

Table 1. Split-plot Experimental Design with Time and Temperature as Whole Plot Factors and the Concentration of H_2SO_4 as the Split Factor

				Rep. 1		Rep. 2	
Temp. (ºC)	[H_2SO_4] (%)	Time (min)	Treatment	Run	Day	Run	Day
T1=105 (5 psi)	C0 = 0	t1 = 15	1	2	1	28	5
		t2 = 30	5	3	1	25	5
		t3 = 45	9	17	3	29	5
	C1 = 0.5	t1	2	13	3	21	4
		t2	6	19	4	32	6
		t3	10	14	3	66	11
	C2 = 1.25	t1	3	5	1	58	10
		t2	4	12	2	33	6
		t3	13	15	3	35	6
	C3 = 2.0	t1	17	20	4	48	8
		t2	21	1	1	50	9
		t3	14	7	2	40	7
T2=120 (15 psi)	C0 = 0	t1	18	43	8	55	10
		t2	22	9	2	47	8
		t3	25	38	7	64	11
	C1 = 0.5	t1	23	54	9	69	12
		t2	33	27	5	44	8
		t3	7	30	5	63	11
	C2 = 1.25	t1	11	11	2	71	12
		t2	8	6	1	49	9
		t3	12	68	12	36	6
	C3 = 2.0	t1	15	24	4	60	10
		t2	19	4	1	46	8
		t3	23	16	3	52	9
T3=135 (32 psi)	C0 = 0	t1	16	41	7	65	11
		t2	20	45	8	61	11
		t3	24	8	2	34	6
	C1 = 0.5	t1	26	31	6	72	12
		t2	30	22	4	56	10
		t3	34	59	10	67	12
	C2 = 1.25	t1	27	18	3	39	7
		t2	31	26	5	70	12
		t3	35	51	9	53	9
	C3 = 2.0	t1	28	37	7	57	10
		t2	32	10	2	42	7
		t3	36	23	4	62	11

To account for evaporation, flasks were weighed before and after hydrolysis and the weight difference was assumed to be the result of evaporated water. Deionized water equivalent to this difference was added to standardize the volume back to the initial 250 mL.

Fermentable and Total Sugars Analysis

Soluble sugars in the liquid fraction were determined by High-Performance Size Exclusion Chromatography with Refractive Index (HPSEC-RI) detection according to Giannoccaro *et al.* (2008). The equipment was a Waters HPSEC-RI (Milford, MA, USA) system consisting of a 515 HPLC pump with an injector valve with a 200 µL sample loop, an in-line degasser, and a 2410 refractive index detector maintained at 40 ºC. Sugars were separated using two columns: a Shodex OH Pack SB-804 HQ (300 x 8 mm) connected in series to a Shodex OH Pack SB-802 HQ (300 x 8 mm) (Showa Denko America, Inc., New York, NY, USA). Both columns were maintained at 55 ºC with a Waters column heater module model WAT038040. Columns were preceded by a Shodex OH pack SB-G (50 x 6 mm) guard column. The mobile phase was 0.1 M $NaNO_3$ with 0.2% NaN_3 at a flow rate of 0.4 mL/min. Prior to injection, samples were diluted with D.I. water and filtered through a 0.45 µm nylon membrane syringe filter. Then a full-loop injection was made and run for 46 min. Sugars were quantified with 6-point calibration curves for the following sugars: glucose, fructose, sucrose, raffinose, stachyose, maltohexaose, and maltotetraose. Fermentable sugars were calculated by adding maltohexaose, sucrose, glucose, and fructose. Total sugar content in the liquid fraction was determined by the phenol sulfuric acid method using glucose as standard (Dubois 1956).

Analysis of 5-Hydroxymethylfurfural, Furfural, and Acetic Acid in the Liquid Fraction

The concentrations of 5-hydroxymethylfurfural **(**5-HMF) and furfural were determined by HPLC using a Shimadzu C-18 column (50 mm x 4.6 mm) and UV detection at 284 nm. Compounds were eluted with acetonitrile:water (30:70 v/v) at a flow rate of 0.5 mL/min. The equipment was a Prominence Ultra Fast Liquid Chromatograph with a DGU-$20A_3$ degasser, LC-20AB pumps, a SIL-10AF autosampler with a 50 µL sample loop, a CTO-20A column oven, and an SPD-20 AV UV-visible detector (Shimadzu, Kyoto, Japan). Prior to the analysis, samples were diluted with D.I water and filtered through a 0.45 µm nylon membrane syringe filter. The run time was five minutes with an injection volume of 500 uL.

Acetic acid was analyzed using the method described by McGinley and Mott (2008) with the Waters equipment described in the sugar analysis section and a Rezex ROA-organic acid H+ (8%) (150 x 7.80 mm) column with a guard column KJ0-4282 (Phenomenex, Torrance, CA) maintained in a column heater at 60 ºC. The mobile phase was 0.005N H_2SO_4 at a flow rate of 0.6 mL/min and the run time was 15 min.

Crude Protein Analysis

The crude protein content was determined by the nitrogen combustion method (A.O.A.C. 1990b) using an Elementar Variomax Instrument (Elementar Americas, Inc. Mt. Laurel, NJ, USA). The SBM solid was dried at 80 ºC for 24 h prior to analysis. A 220 mg sample of the dried SBM solid was used for analysis. A numerical factor of 6.25 was used for the conversion from total nitrogen to crude protein.

Color Determination

Color of treated and untreated SBM was determined with a Minolta Chroma Meter CR-400 using the granular materials attachment CR-A50 (Minolta Camera Co. Ltd, Osaka, Japan). The Chroma Meter was calibrated with a white tile and black card before each use.

Cellulose Degradation in the Hydrolyzed Soybean Meal

To determine surface degradation of SBM particles after the hydrolysis treatments, images of selected samples were taken with a scanning electron microscope (SEM FEI ESEM XL-30. Philips, USA.). Samples were gold-coated with a sputter coater (EMITECH, SC7620, Quorum Technologies Ltd, UK.) prior to imaging. Additionally, cellulose degradation was evaluated using a method described by Updegraff (1969). Following the removal of lignin, hemicellulose, and xylosan materials with acid/nitric acid reagent, the cellulose was hydrolyzed with 67% sulfuric acid. The cellulose concentration was determined by the anthrone method using a cellulose calibration curve.

Statistical Analysis

Data were analyzed by conducting an ANOVA test with SAS Version 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Differences in the means for yield of fermentable sugars, HMF, furfural, and color at the end of the acid hydrolysis were analyzed by Fisher's least significant difference procedure with $\alpha = 0.05$.

Cellulose degradation data were analyzed with JMP® Version 9.0.0 (SAS institute Inc., Cary, NC, USA). Analysis of variance and Tukey-Kramer test ($\alpha = 0.05$) were also carried out to analyze differences in the mean cellulose composition at following acid hydrolysis.

RESULTS AND DISCUSSION

Composition of Untreated Soybean Meal

The results of the proximate analysis on w/w dry basis of the untreated SBM were as follows: 48.72 ± 0.01% crude protein, 22.15 ± 0.07% total soluble sugars, 2.80 ± 0.04% starch, 4.32 ± 0.01% acid detergent fiber, 15.64 ± 0.01% neutral detergent fiber (which includes cellulose and hemicellulose), and 5.87 ± 0.02% ash. In addition, the untreated SBM contained 7.91 ± 0.21% d.b. fermentable sugars. These results are comparable to other SBM analyses reported previously by other authors (Grieshop *et al.* 2003; Dale *et al.* 2009; da Silva *et al.* 2009).

Fermentable Sugars in the Liquid Fraction

The content of fermentable sugars extracted from the solids that ended up in the liquid fraction was affected by all main factors and interactions ($p<0.05$). The treatment at 135 °C, 2.0% H_2SO_4, and 45 min (T3C3t3) produced the highest amount of fermentable sugars, 32.2 g per 100 g of initial SBM in d.b., which is a significant increment from the untreated meal that contains 7.9% fermentable sugars in d.b. (Fig. 1).

Glucose and fructose were the fermentable sugars with the highest concentration in most treatments and were likely generated as a result of the breakdown of sucrose, stachyose, and raffinose as well as cellulose and hemicellulose originally present in the SBM.

The highest glucose concentration (19.2% d.b.) was obtained at 135 °C, 2.0% H_2SO_4, and 45 min (T3C3t3), while the maximum for fructose (8.2% d.b.) was reached at 105 °C, 2.0% H_2SO_4, and 30 min (T1C3t2) and also at 120 °C, 2% H_2SO_4, and 15 min (T2C3t1).

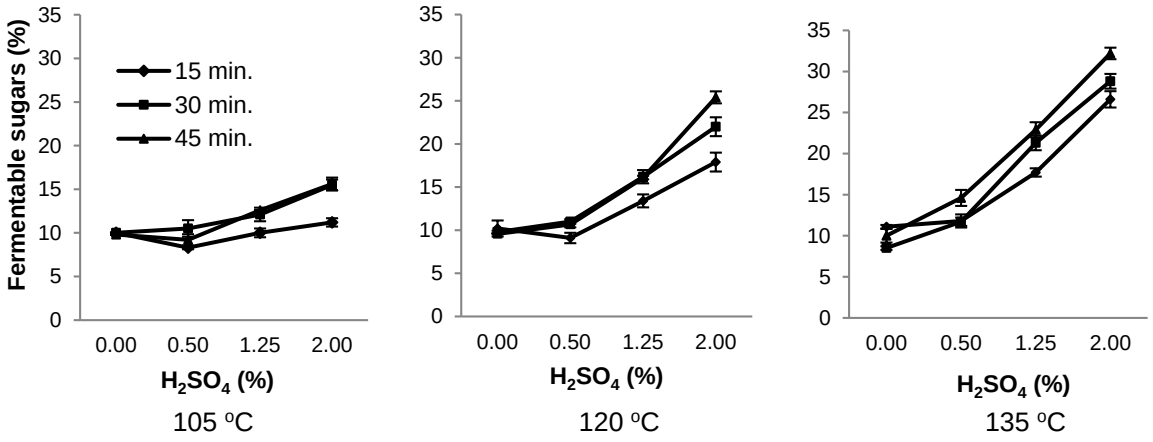

Fig. 1. Fermentable sugars, in grams per 100 grams of untreated soybean meal in dry basis. Untreated SBM had a fermentable sugar content of 7.9 g/100 grams d.b. The LSD used to compare fermentable sugar means for values at different temp*time combinations was 1.53%; for values at different concentrations (with same temp*time combination) it was 1.45%.

The other fermentable sugars after the hydrolysis treatments were sucrose and maltohexaose. For the range of temperatures studied, production of sucrose declined as the concentration of sulfuric acid increased, which is probably the result of its hydrolysis into fructose and glucose. Production of maltohexaose, on the other hand, was favored by increasing concentrations of sulfuric acid for all temperature and time ranges (data not shown).

Production of fermentable sugars in the liquid fraction increased with the severity of hydrolysis conditions due to the breakdown of cellulose. Evidence of cellulose degradation was demonstrated by analyzing untreated soybean meal and the solids after hydrolysis for all factors combinations. At 105 ºC there was no significant change in the percentage of cellulose in the samples. However, as conditions became more severe, cellulose degradation was evident. At 135 ºC and 45 min hydrolysis the end cellulose content was 4.7, 4.4, and 3.75% d.b. for 0.5, 1.25, and 2% acid, respectively. As a reference, the initial content of cellulose in the untreated soybean meal was 7.75%. Hydrolysis at 105 ºC did not show a significant difference in cellulose content in relation to untreated meal.

Scanning electron microscope images showed that SBM particles treated with sulfuric acid underwent a progressive change as the concentration of sulfuric acid increased (Fig 2). Surface particles from the untreated SBM (Fig. 2a) exhibited smooth and clean outer layers, whereas surface particles from the highest treatments (Figs. 2b and 2c) were dispersed with micro-particles and irregular surfaces. This may be evidence that cellulose fibers were agglomerates of individual cellulose micro-fibers, as was reported by Corredor (2008) in soybean hulls. However, because the neutral detergent fiber content was only around 15%, Fig. 2 cannot be taken as a strong evidence of cellulose degradation.

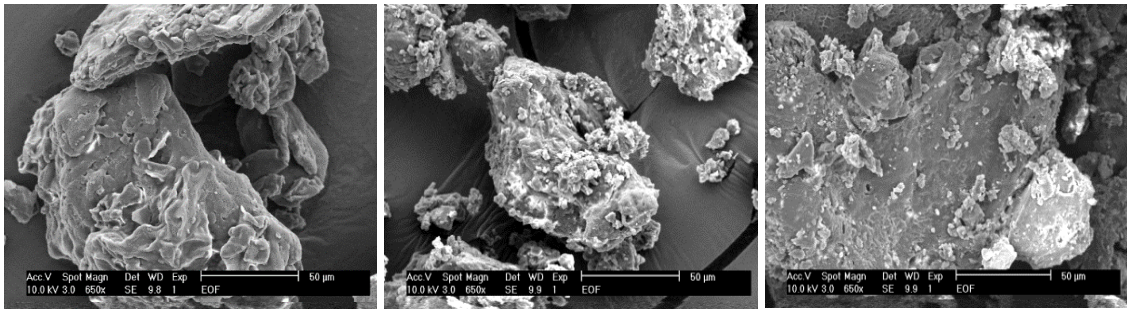

Fig. 2. Scanning electron microscope images of soy bean meal treated with dilute acid at high temperatures. a) Original SBM (No treatment), b) SBM after treatment at 135 °C with 0.5% H_2SO_4 for 45 min, c) SBM after treatment at 135 °C with 2% H_2SO_4 for 45 min.

Crude Protein in the Solid Fraction

The crude protein content in the solid fraction after hydrolysis was affected by all three main factors and the interaction between temperature and concentration of sulfuric acid ($p<0.05$). According to the Fisher's LSD test, of 66 time-temperature combinations, 49 were significantly different. The remaining interactions and the block effect were not significant ($p< 0.05$). The highest protein content was 58.6% d.b. for the treatment at 120 °C, 0.5% H_2SO_4, and 45 min (T2C1t3). This represents a 10-percentage-point increase from the untreated meal that contained 48.1% d.b. of crude protein. In contrast, the treatment at 135 °C, 2.0% H_2SO_4, and 45 min (T3C3t3) lowered the content of protein in the solid fraction by 2 percentage points.

A comparison between protein content in the solid fraction and fermentable sugars in the liquid fraction showed that hydrolysis conditions at the lowest levels of the three factors increased the protein content of the solid fraction without a significant increase in the production of fermentable sugars (Fig. 3, square markers).

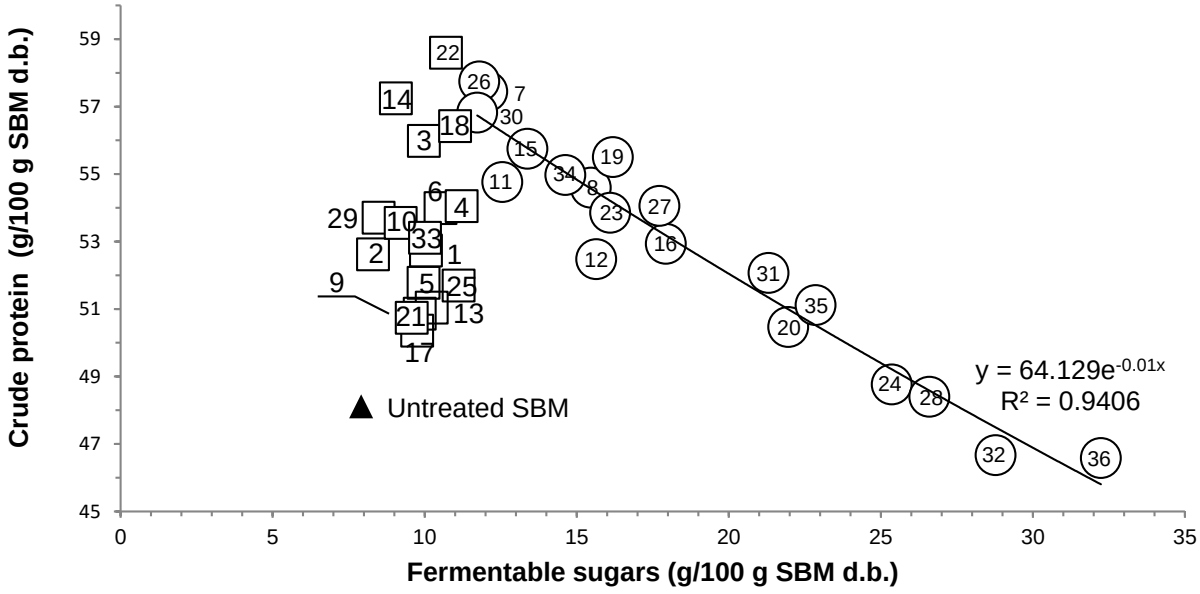

Fig. 3. Crude protein content as a function of fermentable sugars produced. Numbers in markers indicate hydrolysis conditions presented in Table 1.

As hydrolysis conditions became harsher (higher temperatures, concentrations of acid, and durations), the protein content in the solids decreased progressively as fermentable sugars increased (Fig. 3, round markers). The reduction of protein content was likely the result of protein hydrolysis that produced small peptides and other nitrogen compounds that were removed from the solid fraction and transferred to the liquid.

5-Hydroxymethylfurfural, Furfural, and Acetic Acid

Production of 5-HMF and furfural increased as concentrations of fermentable sugars increased (Fig. 4). The maximum levels of 5-HMF and furfural were 0.002 g/L and 0.32 g/L, respectively, with treatment 135 °C, 2% H_2SO_4, and 45 min. (T3C3t3). The maximum 5-HMF concentration was lower than the concentration (0.7 g/L) reported by Panagiotopoulos *et al.* (2012) after dilute acid treatments in barley straw; however, Saha *et al.* (2005a) did not detect any 5-HMF or furfural when they hydrolyzed rice hull with dilute H_2SO_4 (1% v/v) at 120 to 190 ºC. Likewise, Saha *et al.* (2005b) did not find measurable amounts of 5-HMF in wheat straw hydrolyzed with dilute H_2SO_4 (0.5% v/v) at 180 ºC for 15 minutes, but they observed furfural (32 mg/g wheat straw d.b.) and acetic acid (24 g/g wheat straw d.b.).

The highest acetic acid concentration (Fig. 4) was generated by the most severe treatments—135 °C, 2% H_2SO_4, and 45 min. (T3C3t3) and 135 °C, 2% H_2SO_4, and 30 min (T3C3t3) (0.87 and 0.85 g/L, respectively)—and no acetic acid was observed in the mildest treatments. Acetic acid is mainly formed from acetylated sugars derived from hemicellulose (Taherzadeh and Karimi 2007; Larsson *et al.* 2000). Thus, the acetic acid concentration is augmented by increasing severity of hydrolysis conditions, since the same trend was noted for fermentable sugars and hemicellulose/cellulose degradation.

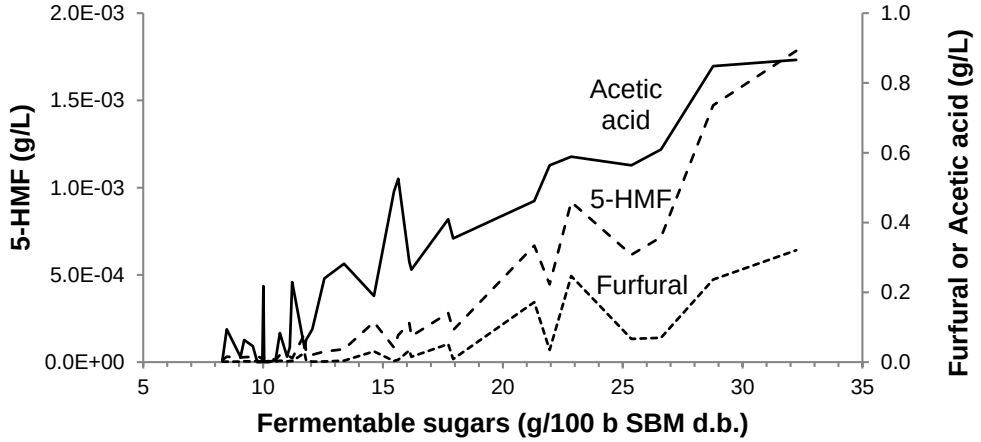

Fig. 4. Formation of fermentation inhibitors 5-HMF, furfural, and acetic acid with increasing contents of fermentable sugars

Color

With the exception of the interaction of concentration*time, concentration* temperature, and block interactions; all other terms had a significant effect on the *L**, *a**, and *b** values in the solid fraction after hydrolysis ($p<0.05$). When the color values were plotted against sugar concentration, *L**-value exhibited the highest decline as sugar concentrations increased, which follows increasing levels of time, temperature, and concentration of acid and is indicative of darkening of the material (Fig. 5). The change in

*L** value followed a first-order decline, which is typical of color changes in many food products (Ling *et al.* 2014). Although there were changes in *a** and *b** values as sugars increased, the changes were not as important as in the case of *L** values. This is an indication of a lesser effect of the treatments on the green and red scales. Of all treatment combinations, *a** and *b** values were statistically different in 58.3 of the cases according to the Fisher's test applied in all mean differences ($p<0.05$).

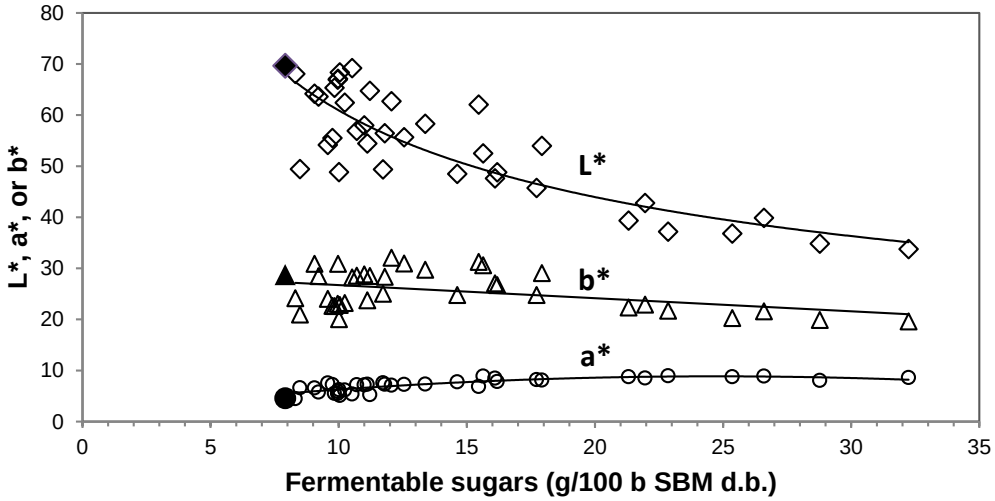

Fig. 5. Means of color values for solid fraction after hydrolysis. *L**-value (0=black and 100= white), *a**-values (-*a**= green, +*a**= red), *b**-values (-*b**= blue, +*b**= yellow). Solid markers indicate the color coordinates for untreated soybean meal

CONCLUSIONS

1. The conditions that maximize the protein content in the solid fraction and the sugars content in the liquid fraction were not in the same range. As the concentration of sugar increased above 11 g/100 g of SBM d.b., crude protein content decreased, indicating protein degradation, likely due to Millard type reactions. Following an opposite trend, the fermentation inhibitors 5-HMF, furfural, and acetic acid sharply increased as the concentration of sugars rose.
2. Fermentable sugars obtained after hydrolysis increased with increasing levels of temperature, time, and concentration of acid. Treatments that produced fermentable sugars—in the liquid fraction—in the range of 8 to 11 g/100 g of SBM d.b. generated protein contents between 50 and to 58 g/100 g of untreated SBM in d.b.
3. Color values were affected with increasing concentration of sugars. Color parameters *a** and *b** varied within narrow ranges as concentration of sugars rose; however, *L** values declined from 69.6 (untreated meal) to 33.8 for the treatment with the longest duration and highest temperature and concentration of acid.
4. If the premise is to obtain the highest amount of fermentable sugars without regard the protein content, then the treatment at 135 °C, 2% H_2SO_4, and 45 min (T3C3t3) produced 32.2% d.b. of fermentable sugars and generated relatively low 5-HMFand furfural levels (0.0018 g/L and 0.32 g/L, respectively). On the other hand, the level of acetic acid, 0.87 g/L, was the highest.

5. If the premise is to maximize the amount of protein, then the treatment at 120 °C, 0.5% H_2SO_4, and 45 min would boost the amount of protein from 48.1% d.b. (untreated SBM) to 58.6% d.b. without considerably altering the SBM original color; however the level of fermentable sugars would be just 10.7 g/100 g of SBM d.b. When both sugars and protein levels are important, the treatment at 120 °C, 1.5% H_2SO_4, and 30 min (T3C2t2) achieved the best balance between a high concentration of fermentable sugars (21.3% d.b.) in the liquid fraction and crude protein (52.1% d.b.) in the solid fraction without a significant change in the original color of the solid fraction.

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