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Comparison and optimization of enzymatic saccharification of soybean fibers recovered from aqueous extractions

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ABSTRACT

Soybean insoluble fractions recovered from aqueous extraction processing (AEP) and enzyme-assisted AEP (EAEP) of full-fat soybean flakes (FFSF) and extruded FFSF were evaluated as a feedstock for the production of fermentable sugars using enzymes. Among the four insoluble fractions (AEP FFSF, EAEP FFSF, AEP extruded FFSF and EAEP extruded FFSF), the composition analysis revealed that the one recovered from EAEP of extruded FFSF had the highest glucan content, 16% [dry basis (db)], as compared to about 10% (db) for the other fractions. Thirty-three percent of the initial glucan of the insoluble recovered from AEP and EAEP of FFSF were converted into glucose using 33 FPU of Accellerase 1000/g-glucan. This saccharification yield was increased to 44% with extruded fibers. The higher saccharification yield of 49% was obtained at 45 °C, 1% glucan loading, and 101 FPU/g-glucan enzymes loading after 27 h of hydrolysis.
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1. Introduction

The main source for biorenewable energy has been corn, sugarcane, switchgrass and woody biomass, while other sources such as algae and fungi are currently being considered and developed. Soybeans, with 3.4 billion bushels produced in 2009, are the primary oil seed grown in the United States (USDA-NASS, 2010). Extensive research has been done on soybean oil for biodiesel production but interest on its cellulosic material as a source of fermentable sugars has been limited to a few studies and therefore its potential is not yet fully understood. Some studies have explored the potential of soybean material as a fermentation source for acetone, butanol or ethanol production (Qureshi et al., 2001; York and Ingram, 1996), and soybean stalk and soybean straw were studied for the production of value-added chemicals (Xu et al., 2007, 2006). A couple of recent studies have focused on the potential of soybean hulls as a source of fiber for production of fermentable sugars (Corredor et al., 2008; Karuppuchamy and Muthukumarappan, 2009) and ethanol production (Mielenz et al., 2009). Pretreatments applied to soybean materials include dilute acid, modified steam-explosion and extrusion. These pretreatments are required for optimum conversion yields. The key component in ethanol production from cellulosic material is indeed providing accessibility of the saccharification enzymes to their substrates, which involves chemical-driven and energy-consuming pretreatment, often resulting in the production of toxic waste.

Soybean oil is produced mainly via solvent (hexane) extraction method. However, due to restrictive environmental regulations and safety concerns over the use of hexane, an interest in aqueous extraction processing (AEP) has occurred in recent years. Major progress in this technology has been recently done by a research team at Iowa state university that has developed an environmentally friendly alternative to solvent extraction (de Moura et al., 2008, 2009; Jung and Mahfuz, 2009; Jung et al., 2009; Lamsal et al., 2006). To compete with extraction yield obtained with hexane and limit water usage, soybean flakes are submitted to an extrusion pretreatment followed by a two-stage enzyme-assisted aqueous extraction process (EAEP) that recycles both water and enzyme sources (de Moura and Johnson, 2009). One of the co-products of this process is the insoluble fraction, composed essentially of soybean fiber; and the central idea behind this research is that this fraction can be a valuable source for bio-ethanol production. In this study, we hypothesized that the disruption of the cellular integrity of cotyledon cells by flaking or extrusion applied as a pretreatment to improve soybean oil extractability should contribute to facilitate access of saccharification enzyme to their substrates. Our objectives

Abbreviations: AEP, aqueous extraction processing; EAEP, enzyme-assisted aqueous extraction processing; FFSF, full-fat soybean flakes; RSM, response surface methodology; FPU, Filter paper units; LSD, least significant difference; AIL, acid insoluble lignin; ASL, acid soluble lignin; S.R, solid remaining; NREL, National Renewable Energy Laboratory; db, dry basis.

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were first to determine the impact of pretreatment (extrusion vs. flaking) and presence of proteins and oil (AEP vs. EAEP) on saccharification yield of soybean insoluble and second to optimize the saccharification conditions (temperature, enzyme loading and glucan loading) using a response surface methodology (RSM).

2. Methods

2.1. Full-fat soybean flakes preparation

Full-fat soybean flakes (FFSF) were prepared from variety IA 92M91 soybeans harvested in 2008. The soybeans were cracked with a corrugated roller mill (model 10X12SGL, Ferrell-Ross, Oklahoma City, OK, USA) and aspirated in a multi-aspirator (Kice, Wichita, KS, USA) to separate into meats and hulls fractions. The meats were conditioned to 60 °C using a triple-deck seed conditioner (French Oil Mill Machinery Co., Piqua, OH, USA). The conditioned meats were flaked by using a smooth-surfaced roller mill (Rosskamp Mfg Inc., Waterloo, IA, USA) to approximately 0.3 mm thickness and 3–5 mm width. For satisfactory extrusion, the FFSF were adjusted to a moisture content of 15.0% with a Gilson mixer (model # 59016A, St. Joseph, MO, USA). The FFSF were then placed into double polyethylene bags and kept at 4 °C until used.

2.2. Extrusion and aqueous extraction process

The extrusion was carried out on Micro ZSE-27 twin-screw extruder (American Leistritz Extruders, Somerville, NJ, USA) as previously described (Jung et al., 2009). About 300 g of FFSF or extruded FFSF were transferred to a 3 or 4 L jacketed reactor, with bottom drain valve (Chemglass, Vineland, NJ, USA), along with water to achieve 1:10 flakes-to-water ratio [dry basis (db)]. The temperature was raised to 50 °C via a circulator (HAAKE Phoenix P1, Thermo HAAKE, Portsmouth, NH, USA). The pH was adjusted to 9.0 with 2 N NaOH. For EAEP, Protex 6L (P6L), an alkaline serine endo-peptidase with optimal pH 9.5 and optimal temperature 60 °C, from Genencor, a Division of Danisco (Rochester, NY, USA), was added to the slurry at 0.5% w/w [g enzyme (as is)/g flakes (db)]. The slurry was stirred via external stirring (600 rpm) (LR 400C, Fisher Scientific, Fair Lawn, NJ, USA or BDC3030, Caframo, Wiarton, Ontario, Canada) for 1 h. The pH of the slurry was kept nearly constant by the addition of 2 N NaOH. The same operation without enzyme addition was referred to as AEP.

2.3. Fractionation

The slurry was centrifuged at 3000 × *g* for 15 min at 20 °C (Avanti J-20 XPL, Beckman Coulter, Fullerton, CA, USA) to separate the insoluble fraction from the liquid fractions (skim, cream, and free oil), which were discarded. The insoluble fractions were stored at 4 °C for up to 3 weeks before use.

2.4. Carbohydrate, lignin, oil, protein and solid determinations

The carbohydrates and lignin contents (acid soluble and acid insoluble lignin) in the FFSF, extruded FFSF and insoluble fractions were determined by the National Renewable Energy Laboratory (NREL) Chemical Analysis and Testing Standard Procedures NREL/TP-510–42618 (NREL, 2008a). Total nitrogen content was determined with Dumas method (AOAC, 1995 method 993.13) in a combustion type nitrogen analyzer (vario MAXCN Elementar Analysensysteme, Hanau, Germany) and conversion to total protein content was performed using a 6.25 conversion factor. Total oil content was determined with the Mojonnier method (AOAC, 2000 method 922.06) and total solid was determined by drying approximately 2.0 g of the sample in a forced convection oven at 130 °C for 3 h. All analyses, except oil content that was determined in duplicate, were performed in triplicate.

2.5. Enzymatic digestibility of insoluble fraction

For the preliminary experiment, a certain amount of soybean insoluble [based on 1.0% (w/w) glucan loading] resulting from EAEP and AEP extractions of FFSF and extruded FFSF were dispersed in 35 ml of 0.05 M sodium acetate buffer (pH 5.0) and DI water to adjust the total weight to 150 g. The mixture of tetracycline (600 μl, prepared as 10 mg/ml in 70.0% ethanol), and cycloheximide (450 μl, prepared as 10 mg/ml in DI water) was added to inhibit the microbial growth during enzymatic hydrolysis. The slurry was stirred with a Tornado IS6 Overhead Stirring System (Radleys Discovery Technologies, Shire Hill, Saffron Walden, United Kingdom) at 150 rpm, and the pH was adjusted to 5.0 with 2 N HCl. The temperature was raised to 50 °C. Enzyme Accellerase 1000 (optimal pH 4.0–5.0, optimal temperature 50–65 °C) from Genencor, was added with enzyme loading of 32.6 filter paper units (FPU)/g-glucan [average activity: 66.6 FPU/ ml]. Aliquots of 3 ml of the slurry were taken after 3, 6, 9, 24, 27, 30, and 48 h of saccharification and immediately heated at 75 °C for 10 min in a water bath and then cooled for 10 min in an ice bath. After centrifugation at 8000 × *g* for 10 min (Micro-Centrifuge model 59A, Fischer Scientific, CT, USA), pellets were discarded and supernatants were collected. The supernatants were analyzed for sugar contents as described in Section 2.8. and saccharification yield was calculated as:

$$\text{Saccharification yield}(\%) = \left[\frac{\text{glucose produced(g)x 0.9}}{\text{initial glucan content in the slurry(g)}}\right] \text{x100}$$

where 0.9 is the hydration factor of glucan-to-hydromonomer, i.e., glucose (Chen et al., 2009).

All experiments including extraction and enzyme saccharification treatments were duplicated.

2.6. Determination of Accellerase 1000 activity at different saccharification temperatures

The slurries of insoluble fractions from EAEP of extruded FFSF were prepared as described in Section 2.5. The saccharification reactions were conducted at three different temperatures, 45, 50, and 65 °C. Aliquots of 3 ml were taken after 0, 1, 3, 24, and 48 h of saccharification and centrifuged at 8000 × *g* for 10 min (Micro-Centrifuge model 59A, Fischer Scientific, CT, USA). The pellets were discarded and supernatants were diluted to enzyme concentrations of 5.0 and 3.8 μg/ml with 5.0 mM citrate buffer. The activity of the enzyme Accellerase 1000 was measured by following the NREL Chemical Analysis and Testing Standard Procedures NREL/TP-510-42628 (NREL, 2008b) to determine the effect of saccharification temperature on its activity.

2.7. Optimization of enzymatic digestibility of soybean insoluble recovered from EAEP of extruded FFSF

The effects of temperature (45–65 °C), enzyme loading (6.5–196.0 FPU/g-glucan) and glucan loading (0.5–1.8%) on the saccharification yield of the insoluble fraction resulting from EAEP of extruded FFSF were evaluated with a central composite design. This experiment was composed of 54 runs, split into blocks of 18. The block of 18 runs included the eight 2^3 factorial points, 6 axial points, a replicate of the axial points associated with temperature and 2 central points. Due to the difficulty to perform the

Table 1
Experimental values and coded levels of the independent variables.

Variable	Symbol	Coded factor levels				
		−2	−1	0	1	2
Temperature (°C)	X_1	25	45	55	65	NA
Enzyme loadings (FPU/g-glucan)	X_2	6.5	53.8	101.1	148.4	196.0
Glucan loading (w/w%)	X_3	0.5	0.8	1.1	1.4	1.8

incubations simultaneously at different temperatures, a split-plot component was added to the experimental design. After the initial experiments were conducted, the design was augmented with 12 additional runs at 25 °C done in blocks of 6, to explore the effects of lower temperature. Table 1 shows the shifted and scaled and unshifted and unscaled values of the independent factors (Xi).

2.8. High performance liquid chromatography (HPLC) analysis

Samples were analyzed for sugars by using an Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA) at 85 °C with a Varian HPLC equipped with a refractive index (RI) detector at 35 °C. Sample injection volume was 20 µl and milliQ water at a flow rate of 0.6 ml/min was used as mobile phase. A sample mix of arabinose, fructose, galactose, glucose, raffinose, stachyose, sucrose, mannose, and xylose was used as a reference.

2.9. Statistical analysis

The General Linear Model, PROC GLM and PROC mixed, in SAS systems (version 9.1, SAS Institute Inc., Cary, NC, USA) were used to determine least significant difference (LSD) values and to fit means model, respectively. The NLME and Lattice package in R (version 2.9.2, R Foundation for Statistical Computing, Vienna, Austria) was used to fit the mixed linear model and create plots of the response surface data.

3. Results and Discussion

3.1. Composition analysis of starting materials and their aqueous-insoluble fiber fractions

The major compounds of the FFSF were protein (39.1%, db), followed by oil (22.2%, db), carbohydrates (20.9%, db) and lignin (10.5%, db) (Table 2). The proximate composition of the insoluble fractions recovered after extraction varied depending on the pretreatment and presence of enzyme. The addition of a protease during aqueous extraction of the FFSF only slightly affected the oil extractability, i.e., remaining oil content, in the insoluble fraction, while it significantly increased the oil extraction yield of the extruded FFSF. This was illustrated by the remaining oil content in the insoluble fraction of 9.8% vs. 18.0% for EAEP and AEP extruded FFSF, respectively, which confirmed the previous findings of de Moura et al. (2008) and Lamsal et al. (2006). For the protein fraction, the increased protein content in the insoluble AEP FFSF vs. AEP extruded FFSF can be attributed to the formation of insoluble aggregates during extrusion (Jung, 2009). These aggregates were resolubilized with the addition of the protease during aqueous extraction, thus decreasing the remaining protein content in the insoluble fraction (39.8% vs. 28.7% for AEP extruded FFSF and EAEP of extruded FFSF, respectively).

The carbohydrate compositions revealed that FFSF were composed of 9.1% glucan, 7.3% galactan, 2.6% arabinan, and 1.9% xylan (db). This distribution was not modified by extrusion pretreatment of the FFSF (Table 2). During aqueous extraction, the majority of the soluble oligosaccharides are recovered in the skim fraction, which were separated from the insoluble fraction by centrifugation. Lower amount of soluble oligosaccharides might still be present in the insoluble fraction but were considered as negligible. After aqueous extraction, supplemented or not with a protease (AEP and EAEP), an increase in the carbohydrate contents in the insoluble fiber fractions was observed when compared to the starting material. Because fermentable sugars potentially come from cellulose and hemicellulose (Shreenath and Jeffries, 2000), a higher amount of glucan and xylan is preferred in biomass for bio-ethanol production. The glucan and xylan content in the insoluble fractions were lower than the reported values of 30.0–50.0% glucan and 9.0–22.0% xylan for other agricultural residues such as corn stover, wheat straw and hardwoods (Mosier et al., 2005), but still represent a good source of fermentable sugars. This fraction, which is similar to the residual fraction obtained from the soy industry after soy protein isolate production, can likely be used as animal feed. However, converting it to fermentable sugars for production of value-added products such as bio-ethanol can increase its economical value. The observed increase in the insoluble fractions compared to the FFSF and extruded FFSF was due to the oil and protein removal (Table 2). For example, the highest increase was observed for the EAEP extruded FFSF insoluble with a glucan content of 16.0% vs. 8.7% for the starting material. These values of proximate composition of fiber fractions are in close agreement with those reported values by Guermani et al. (1992) for okara, a by product of soy milk and tofu production. Similarly Balan et al.

Table 2
Composition of FFSF, extruded FFSF and their insoluble fractions.

	S.R. (%)	Glucan (%)	Xylan (%)	Arabinan (%)	Galactan (%)	Mannan (%)	AIL (%)	ASL (%)	Protein (%)	Oil (%)	Ash (%)
Starting material											
FFSF	100.0	9.1 ± 0.1^{a}	1.9 ± 0.1^{a}	2.6 ± 0.2^{a}	7.3 ± 0.1^{a}	0.0^{a}	$9.0 \pm 0.2^{a,b}$	1.5 ± 0.1^{d}	39.1 ± 0.2^{c}	$22.2 \pm 0.8^{c,d}$	ND
Extruded FFSF	100.0	8.7 ± 0.2^{a}	1.8 ± 0.0^{a}	2.8 ± 0.1^{a}	6.9 ± 0.1^{a}	0.0^{a}	10.6 ± 0.2^{b}	1.0 ± 0.1^{b}	38.8 ± 1.8^{c}	22.7 ± 0.3^{d}	ND
Insoluble fractions											
AEP FFSF	51.5 ± 4.5^{a}	10.3 ± 0.0^{b}	$2.9 \pm 0.0^{a,b}$	5.4 ± 0.1^{b}	9.7 ± 0.2^{b}	1.4 ± 0.0^{b}	11.5 ± 0.2^{b}	1.4 ± 0.1^{c}	27.1 ± 0.0^{b}	26.2 ± 0.0^{e}	4.5 ± 0.0^{a}
EAEP FFSF	$47.4 \pm 2.1^{a,b}$	10.4 ± 0.2^{b}	$2.9 \pm 0.2^{a,b}$	6.4 ± 0.1^{c}	11.5 ± 1.2^{c}	0.0^{a}	25.3 ± 1.1^{c}	9.5 ± 0.1^{e}	23.5 ± 1.5^{a}	20.7 ± 1.4^{c}	ND
AEP Extruded FFSF	46.9 ± 3.0^{b}	9.1 ± 0.5^{b}	$2.3 \pm 0.2^{a,b}$	5.2 ± 0.4^{b}	9.8 ± 0.8^{b}	1.4 ± 0.0^{a}	12.1 ± 1.2^{b}	1.3 ± 0.1^{c}	39.8 ± 0.0^{c}	18.0 ± 0.0^{b}	4.9 ± 0.5^{a}
EAEP Extruded FFSF	27.3 ± 1.2^{c}	16.0 ± 0.7^{c}	5.4 ± 2.4^{b}	8.9 ± 0.6^{d}	16.4 ± 2.2^{d}	$0.5 \pm 0.0^{a,b}$	6.7 ± 3.1^{a}	0.7 ± 0.3^{a}	28.7 ± 0.0^{b}	9.8 ± 0.0^{a}	4.1 ± 2.7^{a}
LSD	4.5	1.0	3.2	0.8	1.8	1.1	3.2	0.1	2.4	1.7	3.5

Data are based on oven-dry weight of biomass. Values are expressed as means and standard deviations.
Means within each column followed by different superscript are significantly different at $p < 0.05$ ($n = 3$).
S.R. corresponded for the insoluble fractions to the percentage of solid (db) remaining after aqueous extractions of the starting materials.

(2009) reported comparable data for defatted cake from soy, peanut, DDGS, canola, sunflower and sesame.

Lignin is one of the major components of lignocellulosic biomass, and interferes with the enzymatic hydrolysis of cellulosic material (Cowling and Kirk, 1976; Kaparaju and Felby, 2010; Kim et al., 2003; Mosier et al., 2005; Mooney et al., 1998) and therefore represents an undesirable compound. The fiber-rich insoluble fractions from AEP and EAEP of FFSF have total lignin content (sum of acid soluble and acid insoluble) of 12.9% and 34.8%, respectively, while AEP and EAEP extruded FFSF fiber-rich insoluble fractions have lignin content of 13.4% and 7.4%, respectively. A lignin removal of 36.7% was obtained during AEP of FFSF while no lignin was removed during EAEP of FFSF. This latter unexpected observation could be due to the adsorption of the protease to the lignin surfaces during EAEP of FFSF resulting in the formation of lignin–protein–complex (LPC) as evidenced by the higher mass balance, i.e., 110.0% for EAEP FFSF insoluble fraction. There are also previous studies reporting adsorption of bovine serum albumin protein in the lignin surfaces and adverse effect of protein in lignin quantification (Balan et al., 2009; Yang and Wyman, 2006). Higher lignin removal was obtained during AEP and EAEP of extruded FFSF; 45.8% and 82.6%, respectively (Fig. 1). The delignification occurring in the extruded fiber could be attributed to the high shear, high temperature during treatment, leading to lignin solubilization (Dale et al., 1999; Lee et al., 2009). The presence of protease seemed to promote lignin solubilization. To determine if these fiber-rich insoluble fractions with various remaining oil and protein content are susceptible to enzyme attack, enzyme saccharifications were tested using a commercial enzyme (Accellerase 1000).

3.2. Enzymatic saccharification

The four fiber-rich insoluble fractions can be divided in terms of saccharification potential into two groups: the AEP and EAEP FFSF on one side and AEP and EAEP of extruded FFSF on the other, having a saccharification yield of 32.0% and 44.0%, respectively, after 48 h of saccharification (Fig. 2). The extrusion pretreatment applied before EAEP, by altering the cellular integrity of the cotyledon cells, was not only beneficial to oil and protein extractability as reported above but also significantly facilitated the accessibility of the saccharification enzyme to cellulosic material. Our results also indicated that the higher quantity of residual protein and oil in the fractions (AEP vs. EAEP extruded FFSF) did not impair saccharification yield. The higher saccharification yield of 44.0% obtained in our study was lower than the 62.5% glucose recovery reported

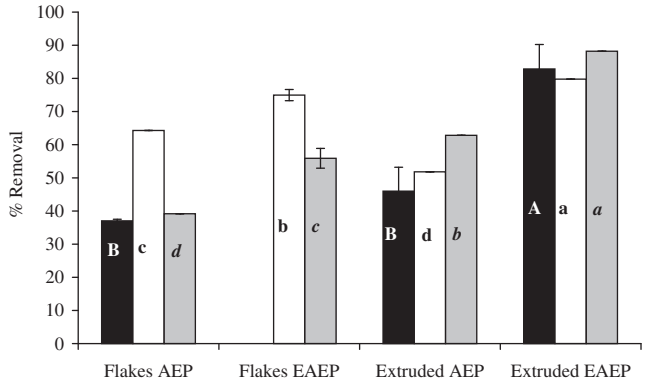

Fig. 1. Percent of compounds removal during AEP and EAEP of FFSF and extruded FFSF, Black bars: lignin; white bars: protein; grey bars: oil. Compound removals sharing the same letters (upper case for lignin, lower case for protein, and italicized lower case for oil) were not statistically different at $p < 0.05$. Removal (%) = $[(X_g - Y_g)/X_g] \times 100$, where, X_g is grams of protein or oil or lignin in flakes or extruded flakes and Y_g is grams of protein or oil or lignin in remaining solids.

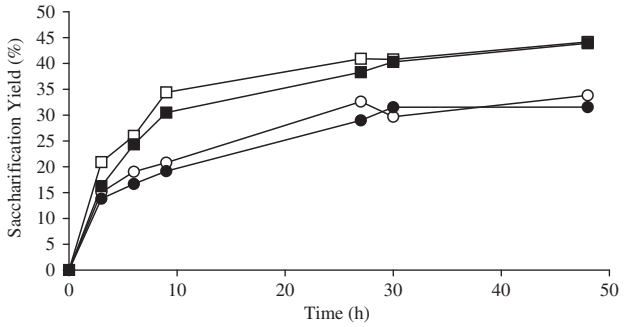

Fig. 2. Effect of extrusion and presence of protease during the extraction on saccharification yield, White circles: AEP FFSF; black circles: EAEP FFSF; white rectangles: AEP extruded FFSF; black rectangles: EAEP extruded FFSF.LSD = 8.34.

from extruded soybean hulls (Karuppuchamy and Muthukumarappan, 2009). This could be attributed to the different extrusion pretreatment conditions and compositional difference between insoluble fiber fraction and soybean hull, which contains 36.0 wt% glucan as reported by Corredor et al. (2008). The potential of combining the extrusion pretreatment with another mild pretreatment is currently being investigated and is expected to increase the saccharification yield. The integration of ammonia fiber expansion (AFEX) and extrusion pretreatment resulted in the glucose yield of 75.0% from soy cakes (Balan et al., 2009).

3.3. Optimization of enzymatic saccharification of insoluble fiber fraction recovered from EAEP of extruded FFSF

A central composite split-plot design was used to identify the best combination of reaction temperatures, enzyme loadings and glucan loadings for optimal saccharification yield of the insoluble fiber fraction of the EAEP extruded FFSF. Saccharification yields at 27 h ranged from 5.3% (65 °C, 6.5 FPU/g-glucan, and 0.5% glucan load) to 50.0% (45 °C, 101.1 FPU/g- glucan, and 1.1% glucan load) (Table 3). With glucan loading of 1.1% at 101.1 FPU/g-glucan enzyme loading, saccharification yield decreased from 50.0 to 36.2 and 22.5% with increasing temperature from 45 to 55 and 65 °C (treatments 6, 12 and 17). The low saccharification yield at high temperature, i.e., 65 °C, could partly be explained by the loss of enzyme activity due to thermal inactivation; Accellerase 1000 activity decreased with increase in temperature during saccharification (Fig. 3). The results depicted that the saccharification yield increased as enzyme loadings increased; saccharification yield increased from 16.0% to 39.0% at enzyme loading of 6.5 FPU/g-glucan and 196.0 FPU/g-glucan, respectively, at 0.5% glucan load and 25 °C (treatments 19 and 21). However, maximum glucose yield (50.0%) was obtained at concentration of 101.1 FPU/g-glucan (treatment 6, Table 3). The decrease in hydrolysis rate at higher enzyme loadings might be due to the lower adsorption efficiency of enzyme at higher loadings or due to the saturation of cellulose surfaces with enzymes (Soto et al., 1994). The saccharification yield decreased from 40.4% to 37.3% at glucan loadings of 0.5% and 1.8%, respectively, and at 196.0 FPU/g-glucan enzyme loading and 45 °C (treatment 3 and 4). The glucan loadings of $\leqslant 1.1\%$ gave the higher saccharification yield indicating that higher glucan load created mixing problem and reduced the aqueous phase in the mixture and thereby hindered enzymatic hydrolysis (Xu et al., 2007).

3.4. Statistical analysis

A response surface quadratic model was fitted to the data to determine the effect of temperature, enzyme loadings and glucan loadings on saccharification yield from EAEP extruded FFSF fibers.

Table 3
Central composite design matrix for the three independent variables in coded values and real values and saccharification yield obtained after 27 h.

Treatment #	Temperature (°C)	Accellerase 1000 loading (FPU/g-glucan)	Glucan loading (%)	Saccharification yield (%)
	X_1	X_2	X_3	
1	−1 (45)	−2 (6.5)	−2 (0.5)	16.3 ± 7.2
2	−1 (45)	−2 (6.5)	2 (1.8)	7.5 ± 2.2
3	−1 (45)	2 (196.0)	−2 (0.5)	40.4 ± 5.5
4	−1 (45)	2 (196.0)	2 (1.8)	37.3 ± 1.3
5	−1 (45)	0 (101.1)	0 (1.1)	48.3 ± 6.2
6	−1 (45)	0 (101.1)	0 (1.1)	50.0 ± 11.7
7	0 (55)	0 (101.1)	−1 (0.8)	40.0 ± 1.9
8	0 (55)	0 (101.1)	1 (1.4)	33.4 ± 3.2
9	0 (55)	−1 (53.8)	0 (1.1)	37.0 ± 6.8
10	0 (55)	1 (148.4)	0 (1.1)	42.0 ± 3.3
11	0 (55)	0 (101.1)	0 (1.1)	38.3 ± 0.9
12	0 (55)	0 (101.1)	0 (1.1)	36.2 ± 4.0
13	1 (65)	−2 (6.5)	−2 (0.5)	5.3 ± 2.7
14	1 (65)	−2 (6.5)	2 (1.8)	15.7 ± 4.2
15	1 (65)	2 (196.0)	−2 (0.5)	23.3 ± 1.7
16	1 (65)	2 (196.0)	2 (1.8)	17.8 ± 2.0
17	1 (65)	0 (101.1)	0 (1.1)	22.5 ± 2.3
18	1 (65)	0 (101.1)	0 (1.1)	28.0 ± 2.4
19	−2 (25)	−2 (6.5)	−2 (0.5)	16.0 ± 1.0
20	−2 (25)	−2 (6.5)	2 (1.8)	11.8 ± 1.3
21	−2 (25)	2 (196.0)	−2 (0.5)	39.3 ± 9.5
22	−2 (25)	2 (196.0)	2 (1.8)	36.5 ± 2.9
23	−2 (25)	0 (101.1)	0 (1.1)	34.1 ± 0.8
24	−2 (25)	0 (101.1)	0 (1.1)	32.0 ± 1.5

Value in parenthesis represents real values.

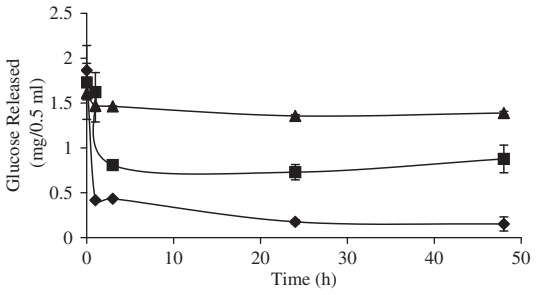

Fig. 3. Effect of saccharification temperature on Accellerase activity. Diamond: 65 °C; rectangle: 50 °C; triangle: 45 °C.

The response surface quadratic model was tested for its significance by a lack-of-fit test (Table 4), which indicated that the model exhibited a lack-of-fit ($p > 0.0001$) and therefore could not be used for the prediction of optimum conditions in the range of evaluated input variables in the experimental design (Table 4). According to Rabelo et al. (2008), "A model with evidence of lack-of-fit cannot be used for optimization purposes; however, it can be used to plot qualitative response surfaces that can aid in determining the best experimental region". Therefore response surface for saccharification yield vs. glucan loading and saccharification temperature at different enzyme loadings of insoluble fiber fractions were plotted to determine the region of higher glucose yield (Fig. 4). Since the quadratic response surface model exhibited a lack-of-fit, means model was fitted to the data to identify the input variable values in the design point, which led to the highest estimated glucose yield. In the means model, each design point has its own mean and the optimum conditions correspond to the design points with the largest mean. Also a confidence region for the optimum conditions is defined by the set of design points whose mean is not significantly different from the largest mean. The means model was highly significant ($p < 0.0001$) at 5.0% significance level (Table 4). The best design point was 45 °C, glucan loading of 1.1%, and enzyme loadings of 101.1 FPU/g-glucan (Table 5). This analysis also identified the temperature as a significant factor in the saccharification process, no design points at 65 °C being in the confidence region. The loss of enzyme activity at 65 °C was certainly part of this result. The higher saccharification yields obtained from means model (not significantly different from the maximum saccharification yield) were in the region of moderate-to-higher enzyme loadings and in the temperature range of 25–55 °C, but glucan load varied from 0.5% to 1.8%, indicating that reaction temperature and enzyme loadings are more crucial than glucan loadings in

Table 4
Results from statistical analysis of models used to optimize glucose yield from extruded EAEP of fiber.

Source	Numerator degrees of freedom	Denominator degrees of freedom	F-statistics	*p*-value
Lack-of-fit	10.0	29.1	5.5	0.0002
Means model	19.0	25.7	20.5	<0.0001

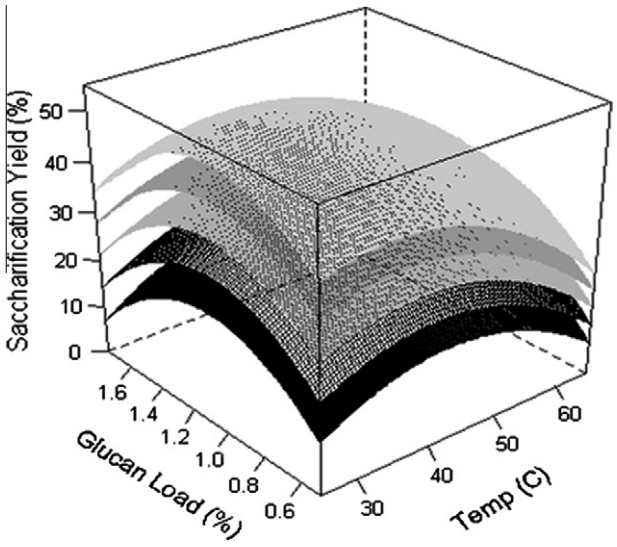

Fig. 4. Effect of glucan load, temperature and enzyme loading on saccharification yield of EAEP extruded FFSF fiber fraction after 27 h incubation. Enzyme loads (FPU/g-glucan) from bottom to top: 6.5 (black); 53.8 (black and grey); 101.1 (grey); 148.4 (dark grey); 196.0 (light grey).

Table 5
Design points with highest saccharification yield from extruded EAEP fiber resulting from means model.

Temperature (°C)	Glucan load (%)	Enzyme load (FPU/g-glucan)	Saccharification yield (%)
Design points with highest mean at 27 h of saccharification			
45	1.1	101.1	49.2 ± 2.6
Design points with means not significantly different from the design point with the highest mean			
25	0.5	196.0	39.3 ± 4.0
25	1.8	196.0	36.5 ± 4.0
25	1.1	101.1	33.1 ± 3.2
45	0.5	196.0	40.4 ± 3.2
45	1.8	196.0	37.3 ± 3.7
55	1.1	101.1	37.3 ± 2.6
55	1.1	53.8	37.0 ± 3.2
55	1.1	148.4	42.0 ± 3.2
55	0.8	101.1	40.0 ± 3.8
55	1.4	101.1	33.4 ± 3.2

the saccharification process. The adsorption of Accellerase 1000 to the lignin is possibly responsible for the need of higher enzyme loadings to obtain higher glucose yield (Kumar and Wyman, 2009). The results of these optimization experiment indicated that the input variable values that optimize the expected glucose yield were not in the experimental design, as evidenced by the lack-of-fit of the quadratic response model.

4. Conclusion

This research has demonstrated the potential use of soybean fiber-rich insoluble fractions recovered from AEP and EAEP of FFSF and extruded FFSF as lignocellulosic feedstock in the production of fermentable sugars via enzymatic hydrolysis. Based on the higher saccharification yield achieved due to extrusion pretreatment of FFSF prior to oil and protein extraction, it could be concluded that further pretreatment methods must be evaluated to optimize saccharification yield. This study has elucidated the importance of soybean fiber as the sugar produced could be converted into bioenergy, biobased products, specialty food and feed ingredients while the residual fiber fractions could be used as animal feed.

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