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Towards complete hydrolysis of soy flour carbohydrates by enzyme mixtures for protein enrichment: A modeling approach

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ABSTRACT

Soy protein is a well-known nutritional supplement in proteinaceous food and animal feed. However, soybeans contain complex carbohydrate. Selective carbohydrate removal by enzymes could increase the protein content and remove the indigestibility of soy products for inclusion in animal feed. Complete hydrolysis of soy flour carbohydrates is challenging due to the presence of proteins and different types of non-structural polysaccharides. This study is designed to guide complex enzyme mixture required for hydrolysis of all types of soy flour carbohydrates. Enzyme broths from *Aspergillus niger*, *Aspergillus aculeatus* and *Trichoderma reesei* fermentations were evaluated in this study for soy carbohydrate hydrolysis. The resultant hydrolysate was measured for solubilized carbohydrate by both total carbohydrate and reducing sugar analyses. Conversion data attained after 48 h hydrolysis were first fitted with models to determine the maximum fractions of carbohydrate hydrolyzable by each enzyme group, i.e., cellulase, xylanase, pectinase and α-galactosidase. Kinetic models were then developed to describe the increasing conversions over time under different enzyme activities and process conditions. The models showed high fidelity in predicting soy carbohydrate hydrolysis over broad ranges of soy flour loading (5–25%) and enzyme activities: per g soy flour, cellulase, 0.04–30 FPU; xylanase, 3.5–618 U; pectinase, 0.03–120 U; and α-galactosidase, 0.01–60 U. The models are valuable in guiding the development and production of optimal enzyme mixtures toward hydrolysis of all types of carbohydrates present in soy flour and in optimizing the design and operation of hydrolysis reactor and process.

1. Introduction

Soybean is the most widely used vegetable protein source for monogastric animals and the most prevalent oil seed crop in the world [1]. Soybean meal, the solid residue after oil is extracted, contains about 50% protein and 30–35% carbohydrate. It is predominantly used as a source of animal protein replacement [2]. In addition, carbohydrate is the predominant component in soybean hulls [3]. Although soybean contains nearly as much carbohydrate as protein, the carbohydrate has so far added much less value due to the complex structures, presence of anti-nutritional factors, and low digestibility by the monogastric animals [4]. The carbohydrate in soybean meal is nonetheless a potential source of fermentable sugars for the production of biofuels and value-added chemicals.

Soy carbohydrate is usually divided into two categories, non-structural and structural, based on their physiochemical properties [3]. Each category makes up approximately half of the total carbohydrate. Nonstructural carbohydrate includes three groups: monosaccharides, oligosaccharides, and storage polysaccharides [5]. The structural polysaccharides, also known as non-starch polysaccharides (NSPs), include dietary fiber components such as cellulose, hemicellulose and pectin [6,7]. Major parts of the mono- and oligo-saccharides are soluble. But the oligosaccharides, when used as animal feed, are reported to depress digestion efficiency. The problem is caused by the absence of α-galactosidase enzymes required for hydrolysis of these oligosaccharides. In addition, the microbial breakdown of oligosaccharides in lower intestine produces gas that causes flatus in rats, humans and swine, and may increase the possibility of diarrhea, abdominal discomfort and nausea [8,9]. The usefulness of structural polysaccharides also depends on their fermentability [10]. Both ruminants and nonruminants are incapable of utilizing the structural polysaccharides without the assistance of microbial enzymes required to break down the polysaccharides[11]. So, hydrolyzing soy oligosaccharides and non-starch polysaccharides is important. It increases the value of soy protein products by increasing their digestibility [12]. In addition, the hydrolyzed sugars may be separated from the proteins and

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fermented to produce biofuel and fine biochemical for novel application [13–15].

Soy carbohydrate hydrolysis requires a complex enzyme system with at least pectinase, xylanase, cellulase and α-galactosidase activities [16]. Fungi have been reported to produce these hydrolytic enzymes. *Trichoderma reesei* and *Aspergillus niger* are among the most extensively studied species [17,18]. For example, Juhasz et al. [19] studied the enzyme production by *T. reesei* RUT C30 on various carbon sources. They reported that the fungus produced an enzyme mixture consisting of cellulases and hemicellulases, the latter include xylanase, mannanase, α-galactosidase, α-arabinosidase, β-xylosidase and acetyl xylan esterase. *A. niger* is a well-known producer of pectinolytic enzymes [20]. While these enzymes can hydrolyze soy carbohydrate, quantitatively describing the hydrolytic efficiency of complex enzyme mixtures on complex solid substrate is challenging. It has been recognized that for fungal enzyme mixtures, a model developed based on only one polysaccharide type does not reliably describe its performance on substrates with multiple polysaccharide types [21]. Many of the previous studies were done with lignocellulosic substrates, where cellulase was the focus enzyme group [22,23]. Soybean meal carbohydrate is different from the common lignocellulosic biomass; the major portion of indigestible soy polysaccharides is pectin and only a small portion is cellulose [24]. Further, even though pectin is the major component, the significant amount of hemicellulose and the small amount of cellulose present are believed to exert significant restraints on the hydrolysis [25]. It is probable that cellulose and hemicellulose restrict the access of pectinolytic enzymes to pectin. Different enzyme groups need to act synergistically to breakdown the complex polysaccharides.

It is obvious from the preceding discussion that studies are needed to better understand the hydrolysis of soy carbohydrate with different enzyme mixtures. As various changes take place concurrently during the course of hydrolysis, an approach to describe the phenomena is by using kinetic modeling. There has been no kinetic modeling study reported for soy carbohydrate hydrolysis. But there have been many mechanistic or empirical kinetic studies done on cellulosic biomass. Kinetic model studies of hydrolysis are helpful for substrate preparation, reactor design, and optimization of feeding profiles of substrate and/or enzyme in a fed-batch operation [26]. Most of the mechanistic models have been conducted using variations of Michaelis–Menten initial velocity kinetics [22]. But the Michaelis–Menten equation, which is developed for enzymatic reactions in solution, may not describe the reactions on insoluble substrates like cellulose and pectin [27]. In this study, we developed an empirical kinetic model for the hydrolysis of soy flour carbohydrate using fungal enzyme mixtures containing various cellulase, xylanase, pectinase and α-galactosidase activities and soy flour concentrations. This model would be helpful for further reactor design, batch and continuous process design and optimization of overall economics.

2. Materials and methods

2.1. Materials and equipment

Defatted soy flour (7B soy flour) and soy hulls were provided by Archer Daniel Midland (Decatur, IL). Water used in the hydrolysis was Milli-Q water (18.2 MΩ-cm at 25 °C; Milli-Q Direct 8, Millipore S.A.S., Molsheim, France). $(NH_4)_2SO_4$ (granular), KH_2PO_4 (99% purity), HCl (concentrated acid, 37.4%) and NaOH (98.8%) were purchased from Fisher Scientific (Waltham, MA). Proteose peptone (from meat, Type I, for microbiology), $MgSO_4{\cdot}7H_2O$ (99%), $MnSO_4{\cdot}4H_2O$ (99%), $ZnSO_4{\cdot}7H_2O$ (ACS reagent grade), $CoCl_2{\cdot}6H_2O$, $FeSO_4{\cdot}7H_2O$ (reagent grade), $CaCl_2{\cdot}2H_2O$ (reagent grade), urea (98%), NaN_3 (>99%) and dinitrosalicylic acid (DNS, 98%) were purchased from Sigma–Aldrich (St. Louis, MO). *A. niger* (NRRL 341), *Aspergillus aculeatus* (NRRL 2053), and *T. reesei* Rut-C30 (NRRL 3469) seed cultures were obtained from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection. Two 3 L Bioflo 110 fermentors (New Brunswick Scientific Co., Edison, NJ) were used for enzyme production by fermentation. Absorbance was measured using a UV/vis spectrophotometer (UV-1601, Shimadzu Corporation, Columbia, MD). The hydrolysis experiments were conducted in a shaker (Thermo Scientific MaxQ 5000 Incubating/Refrigerating floor shaker, Ashville, NC). The centrifuge used was Sorvall Legend X1R from Thermo Scientific (Waltham, MA).

2.2. Enzymes

Commercial cellulase Spezyme CP (Dupont, Cedar Rapids, Iowa) and pectinase (Sigma–Aldrich, St. Louis, MO) were used in addition to the enzyme broths produced in the laboratory. Three different fungal species, *A. niger* (NRRL 341), *A. aculeatus* (NRRL 2053) and *T. reesei* Rut C-30 (NRRL 3469), were used to produce the enzyme broths. The fungal fermentation was performed in a 3 L fermentor containing 1 L of the following fresh medium: soy hulls, 20 g/L; proteose peptone, 1.4 g/L; $(NH_4)_2SO_4$, 4 g/L; K_2HPO_4, 0.32 g/L; KH_2PO_4, 0.21 g/L; and $MgSO_4{\cdot}7H_2O$, 1 g/L. The initial pH was 6.7. Inoculation was done with a pre-grown culture at an initial cell concentration of about 0.1 g/L. Temperature and agitation were maintained at 23 °C and 350 rpm. The pH and DO (dissolved oxygen concentration) were allowed to vary naturally until they dropped to 6 and 20% (air saturation), respectively. Dissolved oxygen concentration (DO) was then maintained at 20% by automatic supplementation of pure oxygen as needed. pH was controlled at 6.0 ± 0.1 by automatic addition of 1 M NaOH or HCl. The fermentation was stopped after 5 days when the enzyme production rate decreased significantly. The enzyme broth used for hydrolysis study was the cell- and solid-free supernatant collected by centrifugation of the fermentation broth at 8000 rpm (9000 × *g*) for 10 min (Sorvall RC 5C, DuPont, Wilmington, DE).

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in 250 mL flasks in a shaking incubator at 50 °C and 250 rpm. Each flask contained 40 mL enzyme broth and an amount of soy flour depending on the designed substrate concentration. Two duplicate control systems, prepared with enzyme-free deionized water, were included in each batch of experiments. Dispersed soy flour in deionized water was warmed to 50 °C. Enzyme broth and then deionized water was added. 1 M hydrochloric acid was used to initially adjust the pH to 4.8. During hydrolysis pH was checked every 4 h and adjusted to 4.8 with 1 M NaOH if required; pH had a slight tendency to decrease during the hydrolysis. Samples were taken at 0, 4, 8, 16, 22 and 48 h in triplicate and heated immediately for 10 min in boiling water to deactivate the enzymes. Samples were then centrifuged to separately collect the solids and supernatant. Supernatants were analyzed for concentrations of reducing sugars and total carbohydrate (methods described in the next section). Total carbohydrate and reducing sugar contents in the soy flour used were separately measured. The total carbohydrate conversion achieved was calculated by dividing the total soluble carbohydrate found in the solution by the total carbohydrate present in the soy flour initially added. The reducing sugar conversion achieved was determined similarly.

2.4. Analytical methods

Cellulase, xylanase and pectinase activities were measured according to the methods reported by Ghose [28]. Bailey et al. [29] and Li et al. [30], respectively. Assay reported by Mukesh Kumar et al. [31] was used to measure the activity of α-galactosidase. Reducing sugar concentrations in the hydrolysates were measured by the dinitrosalicylic (DNS) acid method [32]. DNS solution (3 mL) was placed in a test tube and mixed with 1 mL sample. Then the mixture was heated in a boiling water bath for 5 min. The tube was added with water to 25 mL total volume and cooled to ambient temperature. Absorbance was then measured at 550 nm with a spectrophotometer. The absorbance was converted to reducing sugar concentration according to the calibration curve obtained with glucose solutions as standards. Total carbohydrate concentrations were measured by the phenol–sulfuric acid colorimetric method [33]. A 1 mL sample was mixed with 1 mL aqueous phenol solution (5% w/w) in a test tube, followed by addition of 5 mL concentrated sulfuric acid. 5% phenol in water (w/w) was prepared immediately before the analysis. After 10 min reaction without mixing, the mixture was vortexed for 30 s, cooled to room temperature, and then measured for the absorbance at 490 nm. Blanks were prepared in the identical manner with 1 mL deionized water. Total carbohydrate and reducing sugar contents in the soy flour were determined by first processing the soy flour according to the NREL method [34] and then analyzing the acid hydrolysate by the phenol-sulfuric acid colorimetric method and dinitrosalicylic acid (DNS) method.

2.5. Experimental design

Overall procedure followed in this study included: (1) running many hydrolysis experiments using different enzyme mixtures, soy flour (SF) concentrations, and enzyme-to-SF ratios; (2) fitting all final conversion results to an enzyme saturation-type model to determine the maximum conversions attainable from individual carbohydrate type (hypothetically grouped into pectin, xylan, cellulose, and oligosaccharides) by the corresponding enzyme activity used (pectinase, xylanase, cellulase, and α-galactosidase) and to obtain the best-fit model parameters; and (3) fitting the conversions attained at different hydrolysis time to a kinetic (time-dependent) model that builds on the model parameters obtained from step (2). The