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Enzyme recycle and fed-batch addition for high-productivity soybean flour processing to produce enriched soy protein and concentrated hydrolysate of fermentable sugars

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HIGHLIGHTS

- Advanced enzyme processes enriched protein and separated carbohydrate from soy flour.
- Fed-batch enzyme and flour addition improved monomerization at high solid loading.
- Recycle process retained limiting enzyme to significantly increase monomerization rate.
- High sugar syrup promises value of underused soy carbohydrate as fermentation feedstock.
- Recycle process markedly improved productivities of sugar and protein streams.

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ABSTRACT

Despite having high protein and carbohydrate, soybean flour utilization is limited to partial replacement of animal feed to date. Enzymatic process can be exploited to increase its value by enriching protein content and separating carbohydrate for utilization as fermentation feedstock. Enzyme hydrolysis with fed-batch and recycle designs were evaluated here for achieving this goal with high productivities. Fed-batch process improved carbohydrate conversion, particularly at high substrate loadings of 250–375 g/L. In recycle process, hydrolysate retained a significant portion of the limiting enzyme α-galactosidase to accelerate carbohydrate monomerization rate. At single-pass retention time of 6 h and recycle rate of 62.5%, reducing sugar concentration reached up to 120 g/L using 4 ml/g enzyme. When compared with batch and fed-batch processes, the recycle process increased the volumetric productivity of reducing sugar by 36% (*vs.* fed-batch) to 57% (*vs.* batch) and that of protein product by 280% (*vs.* fed-batch) to 300% (*vs.* batch).

1. Introduction

Biorefinery depends on finding renewable biomass that can be effectively converted to fuels and chemicals. Soybean is well known for its high value oil and protein. It also contains 25–28% carbohydrate (Loman and Ju, 2016a; Medic et al., 2014) which, however, is yet to reach its full value. A few recent studies used soybean processing by-products such as hull, okara, molasses and meal for production of ethanol (Letti et al., 2012; Loman and Ju, 2016b; Mielenz et al., 2009; Schirmer-Michel et al., 2008; Siqueira et al., 2008; Yao et al., 2011) and chemicals (Cheng et al., 2017; Karp et al., 2011; Khare et al., 1995; Thakker et al., 2013; Wang et al., 2015; Wang et al., 2008) by microbial fermentation. These processes could convert only a fraction of the soybean carbohydrate to products. The main obstacle is that the carbohydrate is composed of galacto-oligosaccharides and complex polysaccharides including pectin, hemicellulose and cellulose. Hydrolyzing the carbohydrate to fermentable sugars requires not only pretreatment but also concerted actions of enzymes with wide spectra of activities (Karki et al., 2011; Ouhida et al., 2002). These processes can be more economical if all types of soy carbohydrate can be hydrolyzed and then converted to fermentation products. A recent study modeled the effects of different enzyme activities on hydrolysis of carbohydrate in soybean flour (Loman and Ju, 2016c).

Soybean flour is generated from ground dehulled beans after the extraction of oil. It has about 50% protein and 30–35% carbohydrate (Wang et al., 2004). In a new process (Loman et al., 2016), the carbohydrate was hydrolyzed with enzymes (Li et al., 2017) and

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separated from the remaining solid, which was readily collectable as a protein-enriched product, soy protein concentrate (SPC). The liquid hydrolysate containing hydrolyzed carbohydrate was proposed for use as fermentation substrate. For this use, the hydrolysate needs to have a high carbohydrate concentration, which favors the use of high soybean flour loading in the hydrolysis process. Such a high-loading process has additional advantages such as decreased capital costs due to smaller working volume, lower energy costs for heating (to the reaction temperature of 50 °C) and water vaporization (to concentrate hydrolysate), lower costs to collect/separate product at higher concentrations, and minimized costs for waste and wastewater treatment and disposal (Du et al., 2017; Humbird et al., 2010; Modenbach and Nokes, 2013). On the other hand, previous studies generally suggested that hydrolysis yield would decrease with increasing solid substrate loading (Cara et al., 2007; Geng et al., 2015; Lu et al., 2010). One main reason is the poorer mixing of solid reactants in the reactor. Fed batch processes may be used to manage the solid level throughout the reaction without reducing the overall substrate loading (Gao et al., 2014; Yang et al., 2010; Zhao et al., 2013).

Different fed-batch strategies are possible, depending on the reaction characteristics. The polymeric carbohydrate in soybean flour would first be degraded to soluble oligosaccharides and then further hydrolyzed to monomeric sugars (Loman and Ju, 2016a). The galacto-oligosaccharides present in high concentrations in soybean flour require α-galactosidase for hydrolysis. A previous kinetic study showed that breakdown of solid carbohydrate to soluble oligosaccharides was relatively fast by the combined action of pectinase, hemicellulase and cellulase while the hydrolysis of soluble oligosaccharides and galacto-oligosaccharides to sugar monomers was the rate-limiting step for monomerization of soybean flour carbohydrate (Loman and Ju, 2016c). Carbohydrate monomerization is however crucial to the use of hydrolysate as fermentation substrate (Loman and Ju, 2016b). It is therefore important to improve the conversion rate of soluble oligosaccharides to sugars. It is equally important to increase the sugar concentration in hydrolysate. In earlier studies, even when a high loading of 250 g/L soybean flour was used, the reducing sugar concentration in hydrolysate reached only about 42–45 g/L (corresponding to 65–70% conversion of the carbohydrate in soybean flour, values measured with glucose as standard) (Loman and Ju, 2016a). Hydrolysate recycle may be used to accumulate the sugar concentration. Recycle can be particularly advantageous if the hydrolysate also retains a portion of enzyme for reuse (Lu et al., 2002; Wang et al., 2016). Enzyme recycle can reduce the additional enzyme requirement in the hydrolysis process (Eckard et al., 2013; Tu et al., 2007; Tu et al., 2009). Hydrolysis of all types of carbohydrate in soybean flour requires a wide spectrum of enzyme activities and α-galactosidase was the limiting enzyme in the previous study (Loman and Ju, 2016c). The ideal scenario is to recycle more α-galactosidase in the process, to offset its limitation in hydrolysis rate and achieve faster and more complete monomerization of all types of carbohydrate and increase the sugar concentration of hydrolysate.

In this study, fed-batch and recycle processes were investigated for improving hydrolysis kinetics of soybean flour carbohydrate using an enzyme mixture from *Aspergillus niger* fermentation. For the fed-batch process, different substrate and enzyme feeding profiles were evaluated; for the process with hydrolysate recycle, effects of enzyme loading, retention time of hydrolysis reactor, and recycle rate were evaluated. The following process outcomes were compared: the soluble carbohydrate concentration in hydrolysate, reducing sugar conversion, the enriched protein content in remaining solid product, protein recovery in the soy protein concentrate, and the volumetric productivities of hydrolyzed carbohydrate and protein product.

2. Materials and methods

2.1. Materials and equipment

Defatted soybean flour and soybean hulls were provided by the Archer Daniel Midland Company (Decatur, IL). The soybean flour contained (53.0 ± 1.4)% protein, (26.0 ± 1.3)% carbohydrate (by the phenol sulfuric acid method using glucose as the standard, as described in the Analytical methods section), (9.5 ± 0.7)% moisture, (7.7 ± 0.8)% ash, and (0.9 ± 0.3)% fat. 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) seed culture was 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 a Sorvall Legend X1R from Thermo Scientific (Waltham, MA).

2.2. Enzyme production

Submerged *A. niger* fermentation was performed in a 3 L fermentor containing 1 L fresh medium with the following composition: soybean 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. The medium was inoculated with a pre-grown culture at about 0.1 g/L cell concentration. Temperature and agitation were maintained at 23 °C and 350 rpm. The pH and dissolved oxygen concentration (DO) were allowed to vary naturally until they dropped to 6 and 20% (air saturation), respectively. 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. The broth contained 0.70 ± 0.05 FPU/ml cellulase, 180 ± 5 U/ml xylanase, 7.25 ± 0.42 U/ml pectinase and 8.1 ± 0.3 U/ml α-galactosidase. The broth had very low residual total carbohydrate and reducing sugar concentrations, i.e., 0.40 ± 0.07 g/L and 0.08 ± 0.03 g/L, respectively.

2.3. Batch hydrolysis

Enzymatic hydrolysis experiments were conducted in 250 ml flasks in a shaking incubator at 50 °C and 250 rpm for 48 h. Soybean flour, amount depending on the designed substrate concentration, was dispersed in deionized water and warmed to 50 °C. pH was adjusted to 4.8 by addition of 1 M HCl. 0.5% sodium azide was added to prevent microbial contamination. The fungal fermentation produced enzyme broth was used for hydrolysis at the 1 ml/(g soy flour) enzyme loading. Additional DI water was added to make a total liquid volume of 40 ml. The flask was then placed in the shaker. Samples were taken at regular intervals in