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Soybean hull induced production of carbohydrases and protease among *Aspergillus* and their effectiveness in soy flour carbohydrate and protein separation

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ABSTRACT

Soybean hull consists mainly of three major plant carbohydrates, i.e., cellulose, hemicellulose and pectin. It is inexpensive and a good potential substrate for carbohydrase production because it is capable of inducing a complete spectrum of activities to hydrolyze complex biomass. *Aspergillus* is known for carbohydrase production but no studies have evaluated and compared, among *Aspergillus* species and strains, the soybean hull induced production of various carbohydrases. In this study, *A. aculeatus*, *A. cinnamomeus*, *A. foetidus*, *A. phoenicis* and 11 *A. niger* strains were examined together with *T. reesei* Rut C30, another known carbohydrase producer. The carbohydrases evaluated included pectinase, polygalacturonase, xylanase, cellulase, α-galactosidase and sucrase. Growth morphology and pH profiles were also followed. Among *Aspergillus* strains, morphology was found to correlate with both carbohydrase production and pH decrease profile. Filamentous strains gave higher carbohydrase production while causing slower pH decrease. The enzyme broths produced were also tested for separation of soy flour carbohydrate and protein. Defatted soy flour contains about 53% protein and 32% carbohydrate. The enzymatic treatment can increase protein content and remove indigestible oligo-/poly-saccharides, and improve use of soy flour in feed and food. Protease production by different strains was therefore also compared for minimizing protein degradation. *A. niger* NRRL 322 and *A. foetidus* NRRL 341 were found to be the most potent strains that produced maximal carbohydrases and minimal protease under soybean hull induction.

1. Introduction

Finding inexpensive substrates that induce effective carbohydrase production is important to processing and use of plant materials. Plant biomass contains complex carbohydrate structures that require concerted actions of various carbohydrases to degrade. Production of these carbohydrases depends on presence of effective inducers. Soy is a major crop; 313 million metric tons of soybeans were produced globally in 2015 (Gomes et al., 1995). Soybean hull constitutes 8–10% of the bean weight. It is a major low-value byproduct from soybean processing (Loman and Ju, 2016b). The hull is composed mainly of three types of plant carbohydrate, i.e., cellulose (30–50%), hemicellulose (12–25%) and pectin (6–15%) (Kureshy et al., 2000; Tacon and Metian, 2008; Titgemeyer et al., 1989). It is a good candidate for low-cost renewable feedstock to produce enzymes with wide spectra of carbohydrase activities. In addition, soybean hull has very low lignin content, 1–4% (Kureshy et al., 2000; Tacon and Metian, 2008; Titgemeyer et al., 1989). Pretreatment before use in fermentation is largely unnecessary and only a low amount of recalcitrant residues would remain in the broth produced.

Enzyme production using soybean hull as substrate has so far received little attention. Most studies used *Trichoderma reesei* and *Aspergillus* strains in solid state fermentation (SSF) and evaluated only cellulase and xylanase activities (Biswas et al., 1988; Brijwani et al., 2010; Loman and Ju, 2013; Shah and Madamwar, 2005). For example, Brijwani et al. (Brijwani et al., 2010) studied the production of cellulase (including filter paper activity and β-glucosidase) by SSF with mixed culture of *T. reesei* and *Aspergillus oryzae* using soybean hull and wheat bran as substrate. Gawande and Kamat (Gawande and Kamat, 1999) studied xylanase production by *Aspergillus niger* and *Aspergillus terreus*, respectively, using soybean hull as substrate in both SSF and submerged fermentation. In SSF at 35 °C and 83% moisture, *A. niger* and *A. terreus* produced

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8.7 and 6.2 U/ml xylanase, respectively. In submerged fermentation made in 250 mL shake flasks with 100 mL medium containing 1% soybean hull at 37 °C, the xylanase activities produced by the two species were 24.8 and 12.5 U/ml, respectively. They did not report other enzyme activities. There were no previous studies examining and comparing, among *T. reesei* and different *Aspergillus* species and strains, the more complete profiles of carbohydrase activities produced in submerged fermentation under soybean hull induction.

In this study, *Aspergillus aculeatus*, *A. cinnamomeus*, *A. foetidus*, *A. phoenicis*, and 11 *A. niger* strains were compared, together with *T. reesei* (Rut C30), for soybean hull supported carbohydrase production. The following carbohydrase activities were evaluated: pectinase, polygalacturonase, xylanase, cellulase, α-galactosidase and sucrase. Protease production was also examined, for its importance to a potential use of enzyme mixtures produced. This use was to separate the carbohydrate and protein in soy flour, for improving its use in feed and food. For example, aquaculture industry has been growing rapidly (Little et al., 2016). Fishmeal is the preferred protein source for aquaculture feed (Gomes et al., 1995) but fishmeal production has long reached the sustainable limit (Shepherd et al., 2005). Heightened demand has also caused fishmeal price to rise dramatically (Hardy, 2010). Defatted soy flour/meal contains about 53% protein and 32% carbohydrate (Loman and Ju, 2016b). It offers higher protein content than other plant sources such as wheat, lupin, peas and barley. But this protein content is still significantly lower than that of fishmeal, i.e., 65–72% (Gatlin et al., 2007). Soybeans also contain indigestible oligo-/poly-saccharides that may pose anti-nutritional concerns. It is therefore desirable to enrich protein and reduce indigestible carbohydrate in soy flour. Selective carbohydrate hydrolysis by enzyme may achieve these outcomes (Loman et al., 2016; Loman and Ju, 2016c) by removing hydrolyzed carbohydrate into liquid hydrolysate while keeping protein insoluble near isoelectric pH. The protein-enriched solids can then be readily separated from the hydrolysate. Soy flour carbohydrate is complex, including water soluble sucrose (6–8% soy flour), raffinose (1–2%), stachyose (4–5%) and verbascose (trace), and insoluble polysaccharides (15–18%) such as cellulose (2%), hemicellulose (5%), pectin (10%) and a small amount of starch (0.5%) (Berk, 1992; Karr-Lilienthal et al., 2005; Loman and Ju, 2015). Enzymatic separation of soy flour protein and carbohydrate is therefore a good test for the presence of a complete spectrum of carbohydrases and minimal protease, for selective carbohydrate hydrolysis.

This is the first study that evaluated the soybean hull induced carbohydrase production and compared, among *T. reesei* and various *Aspergillus* species and strains, the relatively complete profiles of carbohydrase activities produced. The fungal enzyme broths were further tested for effectiveness in separating soy flour protein and carbohydrate with minimal protein loss.

2. Materials and methods

2.1. Materials and equipment

All strains used in this study were obtained from Agricultural Research Service Culture Collection of the United States Department of Agriculture. They included *T. reesei* NRRL 11460 (Rut C30), *A. aculeatus* NRRL 2053, *A. cinnamomeus* type strain NRRL 348, *A. foetidus* NRRL 341, *A. phoenicis* NRRL 363, and 11 *A. niger* strains, i.e., NRRL 322, 325, 328, 334, 566, 599, 2270, 3122, 13201, 13219 and 62517. (The *A. foetidus*, *A. phoenicis* and *A. cinnamomeus* strains also belong to the *A. niger* aggregate (Abarca et al., 2004).) Soy hull and flour samples were provided by the Archer Daniels Midland Company (Decatur, IL, USA). Unless specified otherwise, chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). In-house equipment used in the study included a UV–vis spectrophotometer (Shimadzu UV-1601), an orbital shaker with temperature control (Thermo Scientific SHKA5000-7), a centrifuge (Eppendorf Centrifuge 5415D), and a water bath (Boekel Scientific ORS-200).

2.2. Cultivation and enzyme activity comparison

Fungal strains were stored on potato dextrose agar (PDA) at 4 °C and subcultured regularly. To start the cultivation for enzyme production evaluation, the strains were grown on PDA plates for 72 h at room temperature and then the plates were washed with autoclaved deionized water containing 0.05% Tween 80 to collect spores. A fixed spore concentration, 3×10^5 spores/mL, was used for inoculation. All cultivation experiments were made in 250 mL flasks containing 50 mL fresh medium (Coffman et al., 2014) with the following composition: 20 g/L soybean hull, 1 g/L proteose peptone, 0.3 g/L urea, 1.4 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.4 g/L $CaCl_2{\cdot}2H_2O$, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.2 g/L Tween 80, and 1 mL/L trace element solution (5 g/L $FeSO_4{\cdot}7H_2O$, 2 g/L $CoCl_2$, 1.6 g/L $MnSO_4{\cdot}H_2O$ and 1.4 g/L $ZnSO_4{\cdot}7H_2O$). A preliminary study was done to compare sucrose, soy molasses, lactose, and soybean hull as the carbon source. Results (not shown) clearly indicated soybean hull as the best inducing substrate among those tested for carbohydrase production.

Six batches of cultivation experiments were carried out. The cultures were incubated at room temperature in a shaker operating at 250 rpm. Profiles of enzyme activities and culture pH were followed almost daily. The effectiveness of culture broth in separating soy flour protein and carbohydrate and the associated protein loss were also compared. This latter comparison (described more in the next section) was made with supernatants collected by centrifugation (9300*g*) of the culture broths harvested after 72 h cultivation. Duplicate flasks were used for each strain evaluated in a batch of cultivation experiments. More repeated experiments were made for evaluating polygalacturonase, cellulase and xylanase production, pH change, and the soy flour protein-carbohydrate separation effectiveness. These properties were measured in 2–4 repeated batches, with more batches for strains of higher enzyme productivities. In the final batch, production profiles of other enzymes, i.e., pectinase, α-galactosidase, sucrase and protease, were also measured in duplicate systems. Results reported in this work are the averages and standard deviations from all evaluated systems.

2.3. Soy flour protein and carbohydrate separation test

Ten mL of a cell-free enzyme broth (or water for the enzyme-free control system) were diluted with 30 mL water. The solution was adjusted to pH 4.8 and then added to 10 g defatted soy flour. The mixture was reacted for 49 h at 50 °C in a shaker operating at 250 rpm. Samples were taken at 1, 24, and 49 h and centrifuged to separate hydrolysate from the enriched soy protein that remained insoluble at this pH. Hydrolysate was analyzed for the released soluble carbohydrate, as both reducing sugar and total carbohydrate concentrations. The enriched soy protein was weighed and analyzed for protein content.

2.4. Analytical methods

2.4.1. Enzyme activity analysis

As commonly defined, one unit of enzyme activity corresponds to the activity that gives the target product at a rate of 1 μmol/min. In this study the target product concentration was determined by the non-specific 3,5-dinitrosalicylic acid (DNS) test method using different reducing sugars as standards, except for the α-galactosidase and protease assays (details given later). All but the protease assays were done at pH 4.8 and 50 °C, the same as the condition for the soy flour protein-carbohydrate separation test.