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Feeding value improvement of corn-ethanol co-product and soybean hull by fungal fermentation: Fiber degradation and digestibility improvement

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ABSTRACT

Fungal pre-treatment and fermentation could improve nutritional value of wet corn distillers' grains and solubles (WDGS), soybean hull (SH), and their blend in swine and poultry diets, which can potentially increase revenues for corn-ethanol and soybean processing industries while reducing feed cost in swine and poultry production systems. This study evaluated the effectiveness of pre-treatment of SH, WDGS, and their mixture by *Trichoderma reesei* and fermentation by *Aspergillus oryzae* to improve the nutritional profile and digestibility of these ingredients. *T. reesei* produced cellulase and xylanase, resulting in structural carbohydrates reduction by 69.2% while concentrating amino acids from 6.8% to 11% in SH. Fermentation with *A. oryzae* degraded phytate by over 50% and improved *in vitro* digestibility of amino acids in WDGS and its mixture with SH. Fermenting *T. reesei* pre-treated SH by *A. oryzae* showed higher *in vitro* dry matter digestibility than non-fermented substrate. Moreover, the proportion of key amino acids (arginine, threonine, methionine, and lysine) in both *T. reesei* and *A. oryzae* fermented substrates were significantly improved. The results demonstrated the feasibility of combining *T. reesei* and *A. oryzae* in improving feeding value of WDGS and SH for potential use as feed ingredients for monogastric animals.

1. Introduction

Exploring and upgrading potential feeding value of co- and by-products from biofuel and tranditional agricultural industries could provide cost-efficient and high-nutrition feed ingredients to farm animals, thus reducing cost of animal production. Corn (*Zea mays* L.) accounts for 94% of total feedstock used for ethanol production in the U.S., and about 91% of the conversion is *via* a dry-grind process to generate ethanol, CO_2 and co-products (RFA, 2020). One of the primary co-products produced is distillers dried grains with solubles (DDGS) after yeast fermentation and a series of downstream processing (centrifugation and evaporation), and is used as an energy, protein and phosphorous source in animal diets (Han and Liu, 2010). During the dry-grind process, approximately 70% of the dry mass of corn is converted to ethanol and CO_2, resulting in the production of DDGS that contains protein (25–30% dry mass), oil (10–15% dry mass) and fiber (40–50% dry mass) (Han and Liu, 2010). Many newer processes also extract oil from the coproducts for biodiesel production or poultry feed, making proteins even more vital components for DDGS as animal feed. However, the concentrations of several indispensable amino acids such as valine (Val), leucine (Leu), isoleucine (Ile) are in excess, while others such as lysine (Lys), methionine (Met) and threonine (Thr) are deficient relative to the

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requirements of poultry and swine (Spiehs et al., 2002). In addition, the digestibility of fiber in DDGS is less than 20% in the small intestine and less than 50% throughout the entire gastrointestinal tract (Urriola and Stein, 2012), leading reduced energy digestibility and increased animal manure production which causes environmental concerns. Therefore, the inclusion rate of mixing ratio of DDGS to the diet of monogastric animals (pigs and poultry) is limited. Furthermore, phytate is an indigestible phosphorus source commonly found in plant-based feed ingredients. About 40–50% of the phosphorus remaining in DDGS is in the phytate form (Reis et al., 2018). The total tract digestibility of phosphate in DDGS is between 68–73%, higher than it in corn (19–27%) and soybean meal (41–49%) (Almeida and Stein, 2010). But the remaining phosphate is still lost in manure, causing a waste of nutritional value from corn-ethanol plant and swine producer. Phytate can also chelate positively charged cations such as calcium, iron and zinc, and interfere with digestion of other dietary compounds especially protein, lipid and starch (Humer et al., 2015). Soybean hulls (SH) are a co-product from the soybean processing industry, account for 8–10% total mass of soybeans, and have a high concentration of cellulose (40–45%) and hemicellulose (30–35%) with low amounts of lignin (3–4%) (Zhang and Hu, 2012). The total global soybean production by 2030 is predicted to be 371.3 million metric tons with production of 29.7–30.1 million metric tons of SH. SH has been demonstrated suitable as on-farm feeding ingredient for ruminant animals to reduce feeding cost (Ipharraguerre and Clark, 2003). In bioprocessing industry, SH is either hydrolyzed or directly used as substrate to produce lipid (Zhang and Hu, 2012), fatty acids (Cheng et al., 2017), enzymes (Li et al., 2017) and other value-added chemicals such as 2, 3-butanediol (Ourique et al., 2020). Instead of directly treating SH as low-cost livestock feed, there will be greater environmental benefits to add value to SH to encourage greater use in animal feed. This is possible because SH have the potential to induce production of carbohydrate degrading enzymes by several fungi including *Trichoderma reesei* (Li et al., 2017).

Fermentation methods have been reported to improve feeding value of DDGS by co-culturing fungal strains *Aspergillus oryzae*, *T. reesei*, *Phanerochaete chrysosporium* (Lio and Wang, 2012), resulting in a 3.5–15.1% reductions of crude fiber and 1.3–4.2% increase of crude protein content. Bacteria such as *Bacillus subtilis* and *Lactobacillus plantarum* have also been reported to increase protein and total amino acids content, while hydrolyzing macromolecular proteins and lignocellulose into small peptides and monosaccharides, respectively (Wang et al., 2018).

Fermenting and blending SH with corn DDGS has the potential to improve the overall nutritional value of these high fiber feed ingredients. First, SH which are high in fiber content can induce fungal strains to produce carbohydrases (cellulase and xylanase) that degrades fiber and thus, improve energy and nutrient digestibility. Secondly, degradation of fiber can concentrate amino acids in the substrate and therefore improve the total amino acids fraction in the fermented product. Finally, different fungi can be combined simultaneously or sequentially to reduce anti-nutritional factors such as phytate, therefore improving overall digestibility of amino acids, fiber and minerals of the fermented feed. Such integrated pre-treatment and fermentation strategy on SH and DDGS has not been evaluated up to date. In this study, we employed solid-state fermentation with *T. reesei* to treat SH, wet DGS (WDGS), and their mixture. The pre-treated and non-pretreated SH were then mixed with WDGS and fermented by the food-grade fungal strain of *A. oryzae* to further improve the feeding value of the feedstock. Carbohydrase, residue sugars, structural carbohydrates and amino acids were monitored in *T. reesei* treatments, with additional evaluation of phytate, lipid and *in vitro* digestibility in fermentation with *A. oryzae*.

2. Materials and methods

2.1. *Inoculum preparation*

Trichoderma reesei (*T. reesei*) and *Aspergillus oryzae* (*A. oryzae*) were maintained in potato dextrose agar (PDA) plate at 28 °C for 7 days and 4 days, respectively, to optimize development of fungal mycelia and spores. Five pieces of the PDA medium with mycelia (0.5 × 0.5 cm) of each strain were cut and transferred to 100 mL of freshly prepared sterilized (121 °C for 15 min) potato dextrose broth (PDB) medium in each of 250 mL Erlenmeyer flasks. The sub-cultures of *T. reesei* and *A. oryzae* were cultured for 5 days at 28 °C, shaking at 150 rpm for fully development of pelletized mycelia which was used as inoculum for pre-treatment and fermentation.

2.2. *Pre-treatment of SH, WDGS and their mixture by T. reesei*

SH and WDGS both are fiber-rich feedstocks containing about 60% and 37% of structural carbohydrates, respectively, in their biomass (Table 1). The amino acids content in SH and WDGS are 7.6% and 23.7%, respectively (Table 1), lower than it in soybean meal (48%). The high fiber content in SH and WDGS needs to be reduced first by pre-treatment of *T. reesei* which can be induced to produce carbohydrase (Li et al., 2017) that have potential to degrade fiber and concentrate amino acids in SH and WDGS. The first experiment was designed to evaluate the pre-treatment period, substrate weight loss, reducing sugar production, cellulase and xylanase activities of *T. reesei* on SH. A volume of 5 mL sub-culture of *T. reesei* was aseptically inoculated to each fermentation flask containing 10 g of SH (about 90% w/w moisture content) with 16 mL deionized (DI) water. The total moisture content after inoculation was 70% w/w. A control treatment inoculated with 5 mL of sterilized sub-culture (denatured strain) was used for comparison. The fermentation flasks after inoculation were incubated at 28 °C statically for 9, 15, and 20 days. The weights of each empty flask, each flask with substrate after inoculation, and each flask during fermentation at 9, 15, and 20 days were measured to monitor the mass loss during fermentation. The control treatment was incubated under the same conditions as fermentation treatments and stopped after 9 days of incubation. Each treatment was performed in triplicates.

A second experiment involved using SH, WDGS and their mixture (WDGS/SH 75/25) as substrate. The mixing ratio was selected due to the proper aeration, carbon/nitrogen ratio and porous morphology of the substrate mixture that provide appropriate space and surface area for fungal colonization (data not shown). The 10 g of each substrate at dry weight was added to each 250 mL Erlenmeyer flask. The final moisture content of 70% (w/w) in the substrate was achieved by adding deionized water, where the amount of which was based on the moisture content in WDGS (49.1%) and SH (9.2%) and mixing ratio of each substrate. A moisture content of 70% (w/w) of the substrate mixture was selected due to its better growth with fungal strains based on preliminary experiments (data not provided). Three treatments with *T. reesei* were conducted based on the different substrates: SH alone, WDGS alone, WDGS/SH mixture. The prepared substrate was then autoclaved at 121 °C for 15 min to sterilize any possible microbiota existing in the substrate. Inoculum preparation of *T. reesei* was the same as described in Section 2.1. The sterilized substrate was cooled to room temperature prior to inoculation with 5 mL of active pelleted *T. reesei* mycelia from freshly prepared sub-culture. Control treatments (without treatment with *T. reesei*) of the three different substrates were inoculated with the same volume of deactivated *T. reesei* (sterilized at 121 °C for 15 min). The inoculated substrate was hand-shaken gently for a few seconds to properly spread the fungal cells in the sub-

Table 1 – Chemical composition of SH and WDGS.

Feedstock	MC (% w.b.)	TS (% w.b.)	Ash (% d.b.)	CP (% d.b.)	Total amino acids (% d.b.)	Total structural carbohydrates (% d.b.)
SH	6.2 ± 0.1	93.8 ± 0.8	5.6 ± 0.0	10.4 ± 0.7	7.6 ± 1.0	59.7 ± 0.1
WDGS	51.2 ± 0.1	48.8 ± 0.1	4.4 ± 0.1	30.8 ± 0.3	23.7 ± 1.4	36.7 ± 2.0

SH: soybean hull, WDGS: wet distiller's grains and solubles, MC: moisture content, TS: total solids, CP: crude protein, w.b.: wet basis, d.b.: dry basis. All the values are represented as mean ± standard deviation.

strate. All the flasks were incubated statically at 28 °C for 9 days. Each treatment was performed with three replications.

2.3. *Fermentation of WDGS mixing with pretreated and non-pretreated SH using A. oryzae*

The pre-treated SH by *T. reesei* was dried at 60 °C for 48 h and were grounded to pass through a 1 mm sieve (No.18 mesh). Ten grams of WDGS (moisture around 50%) was mixed with 2.5 g (to achieve total dry mass of 7.5 g per treatment) each of the ground *T. reesei* pre-treated SH (WDGS/TR-SH) and non-pre-treated SH (WDGS/SH) in a 250 mL Erlenmeyer flask. For comparison, 15 g of WDGS (7.5 g dry mass) alone was also prepared for fermentation of *A. oryzae*. Deionized water was added to each flask to a level which allowed the total moisture content to reach 70% after 5 mL of *A. oryzae* inoculation. The substrate in each flask was sterilized at 121 °C for 15 min prior to inoculation of *A. oryzae* sub-culture. Non-fermented treatments corresponding to their fermented samples were inoculated with 5 mL of sterilized sub-culture (denatured strain) as a control. The fermentation by *A. oryzae* was performed at 28 °C statically for 5 days. Each fermentation treatment was performed with three replications. All samples after pre-treatment by *T. reesei* and fermentation by *A. oryzae* were collected and dried at 60 °C for 48 h (to achieve moisture of less than 10%) in an air-blow oven to avoid degradation of sugar and amino acids under high temperature. The weight of each empty flask and flask with dried pre-treated or fermented sample were measured to monitor the weight loss of the substrate during the incubation. The dried samples were then ground using a coffee grinder into fine particles and frozen at −20 °C for further analysis.

2.4. *Analytical methods*

2.4.1. *Hydrolytic enzymes, reducing sugar, phytate and lipid*

The crude enzymes were extracted by soaking *T. reesei* pre-treated sample (wet basis) in 0.2 M sodium acetate–acetic acid buffer (pH 4.8) at a ratio of 1:10 (w/v) overnight at 4 °C. Cellulase filter paper activity was determined based on the method reported by Ghose (1987), where the enzyme extracts catalyzed Whatman No.1 filter paper for 60 min to release glucose in a 50 °C water bath. Xylanase activity was determined by catalyzing 1% (w/v) beechwood xylan (Sigma Aldrich, St. Louis, MO, USA) for 5 min in a 50 °C water bath to release xylose (Bailey et al., 1992). Both enzymatic reactions were stopped by adding 3 mL of regular DNS (3,5-dinitrosalicylic acid) solution (containing/L: 10 g NaOH, 2 g phenol, 10 g 3,5-dinitrosalicylic acid, 0.5 g sodium sulfite) followed by immediate heating in boiling (100 °C) water bath for 10 min for color development. The reacting mixture was diluted with deionized water before measuring absorbance in a spectrophotometer (DR 5000, Hach Company, Loveland, Colorado, USA) at 540 nm wavelength. Calibration and calculation of cellulase and xylanase activity were performed based on a method previously reported (Li, 2017).

Reducing sugar of the extracted sample from fermented and non-fermented substrate was determined based on DNS method (Miller, 1959). Phytic acid concentration was determined using Phytic Acid Assay Kit (Megazyme Ltd., Chicago, Illinois, USA) by subtracting free phosphorous (free P) from total phosphorous (total P) in each sample tested. The phytic acid concentration was calculated based on the published method (Megazyme, 2017). Lipids in each sample were extracted based on a previous study (Bligh and Dyer, 1959). The total lipid content was calculated as the weight percentage of lipid per dry sample.

2.4.2. *Structural carbohydrates, amino acids profile and in vitro digestibility analysis*

The structural carbohydrates (glucan, xylan, arabinan, galactan, mannan) content in each sample were determined using a two-step acid hydrolysis method based on NREL method (Sluiter et al., 2008). The prepared sample was filtered with 0.22 μm PTFE filter and analyzed for glucose, xylose, arabinose, galactose, and mannose using HPLC (1200 Infinity Series, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with Biorad Aminex HPX-87 P analytical column (Agilent Technologies), and refractive index detector.

Amino acids in each ground dry sample were released by acid hydrolyzation based on a published method (AOAC, 2005). The hydrolyzed samples were diluted and filtered (0.22 μm PTFE filter) before analysis. Derivatization of amino acids was performed before separation and quantification with HPLC (1200 Infinity Series, Agilent Technologies) equipped with ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 3.5 μm) (Agilent Technologies) and diode array detector using UV light source (Henderson and Brooks, 2010). A total of 17 amino acids: aspartate (Asp), glutamate (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), cysteine (Cys), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys) and hydroxyproline (Hyp) in each sample were separated within 25 min retention time at injection volume of 40 μL, column temperature of 40 °C and total flow rate of 1.5 mL/min.

In vitro digestibility of fermented substrate was achieved *via* a sequential enzymatic hydrolysis using pepsin (P7000, 421 pepsin units per mg solids, Sigma-Aldrich) and pancreatin (P1750, 4 times the specifications of the United States Pharmacopeia, Sigma-Aldrich) to simulate gastric digestion and small intestine digestion of monogastric animals (Jang et al., 2019). The method was modified to suit small sample quantity and large sample size. The *in vitro* dry matter (DM) digestibility was calculated based on the dry mass change of the sample before and after digestion. The amino acid pro-

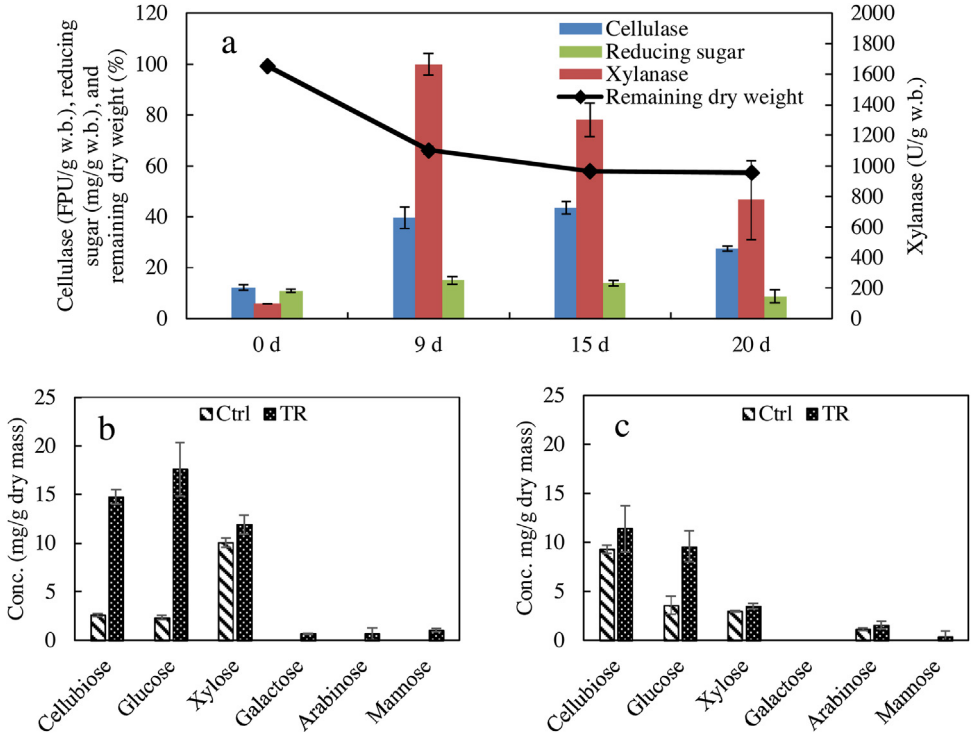

Fig. 1 – Production of cellulase, xylanase and reducing sugars and dry weight loss by *T. reesei* treatment of SH at 0, 9, 15, and 20 days (a); soluble sugar concentration before (Ctrl) and after treatment by *T. reesei* (TR) using SH (b) and WDGS (c) as a substrate. All the values are means with error bars representing standard deviations of three replications.

file of each non-digested and its corresponding digested dry sample were achieved using HPLC method for amino acids as previously discussed. The *in vitro* amino acid digestibility was calculated based on the amino acid content difference between non-digested and digested samples.

2.5. *Statistical analysis*

The statistical analysis was performed with Tukey's multiple comparison of means at 95% confidence interval (p-value <0.05) using JMP Pro 15 (SAS Institute Inc., Cary, NC, USA). The statistical analysis was used to determine pairwise statistical differences ($p < 0.05$) of enzymes activities, structural carbohydrates, amino acids, phytate, lipid, *in vitro* dry matter and amino acids digestibility between each treatment.

3. Results and discussion

3.1. *Pre-treatment of SH and WDGS by T. reesei*

3.1.1. *Effect of T. reesei pre-treatment on structural carbohydrates*

The activities of cellulase and xylanase, concentration of reducing sugars and remaining dry weight of the fermented substrate during 20 days of fermentation by *T. reesei* are depicted in Fig. 1a. The composition of residue sugars in non-pre-treated and 9 d pre-treated SH by *T. reesei* is shown in Fig. 1b. Compared to the beginning (0 d), pre-treatment of SH by *T. reesei* for 9 d showed significant improvement of cellulase by about 2-fold ($p < 0.05$), xylanase by 16-fold ($p < 0.05$), concomitant with increased reducing sugars content by 38% ($p < 0.05$). Continued incubation to 15 d did not show significant ($p > 0.05$) improvement on activities of cellulase, xylanase and concentration of reducing sugar. The dry weight of substrate was reduced significantly by 33.37% ($p < 0.05$) and 41.6% ($p < 0.05$) after 9 d and 15 d fermentation by *T. reesei*, but did not show significant reduction ($p > 0.05$) from 15 d to 20 d fermentation (Fig.1a). These results suggested that 9 d of fermentation of soybean hulls by *T. reesei* was necessary to achieve high activity of cellulase and xylanase, which played a critical role in degradation of cellulose and hemicellulose in the substrate. The residue sugars produced by *T. reesei* after 9 d of fermentation of SH showed a remarkable improvement compared with non-fermented SH. Among them, concentration of cellobiose and glucose in SH after pre-treatment was increased by 4.7-fold ($p < 0.05$) and 6.5-fold ($p < 0.05$), respectively (Fig. 1b). The concentration of xylose increased by 18.4% after SH pre-treatment but the improvement was not significant ($p > 0.05$). Free galactose, arabinose and mannose were also detected in *T. reesei* pre-treated SH, but the improvement in concentration was not significant ($p > 0.05$). The release of glucose and xylose from SH after *T. reesei* pre-treatment were primarily due to enzymatic catalysis by the produced cellulase and xylanase, respectively.

When WDGS was pre-treated by *T. reesei*, only glucose was significantly improved by 1.7-fold ($p < 0.05$) (Fig. 1b), while improvement of cellobiose and xylose was not significant ($p > 0.05$). The soluble sugars from WDGS were lower than those generated in *T. reesei* pre-treated SH. Cellobiose was produced when cellulose and cellodextrins in the substrate were hydrolyzed by endo-glucanase and exo-cellobiohydrolase from *T. reesei*. Cellobiose was further degraded by β-glucosidase into glucose (Coughlan, 1991). The higher improvement of cellobiose and glucose in SH than it in WDGS indicated that higher activity of these above enzymes were present in *T. reesei* pre-treated SH than in WDGS. Because SH had high fiber content than WDGS, it could be indicated that *T. reesei* was efficient in producing fiber degrading enzymes to breakdown fiber into reducing sugars on fiber-rich substrates.

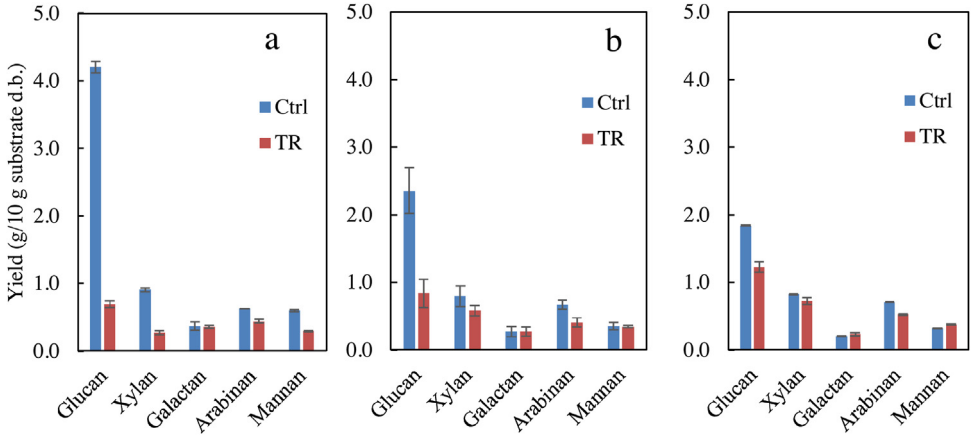

Fig. 2 – Yield of structural carbohydrates in non-pre-treated (Ctrl) and *T. reesei* pre-treated (TR) in (a) SH, (b) WDGS/SH (75/25) mixture and (c) WDGS. The yield of structural carbohydrates considered weight change of substrate before and after pre-treatment. All values are means with error bars representing standard deviations of three replications.

The structural carbohydrates in non-pretreated (0 d) and pretreated (9 d) SH, WDGS/SH mixture and WDGS by *T. reesei* are shown in Fig. 2. Cellulose is comprised exclusively of glucans while hemicelluloses consist of glucans, xylan, galactan, arabinan, or mannan and some acetate. Glucan is a major component of dietary fiber which cannot be digested by mammalian enzymes in small intestine but may be fermented by hindgut bacteria into volatile fatty acids (Urriola et al., 2010). Glucan concentration in WDGS was 18.4% (dry basis) which is similar as the concentration of total dietary fiber in DDGS (18–21% dry basis) as reported (Urriola et al., 2010). Cellulose and hemicelluloses in non-pre-treated SH accounted for 68.2% of total substrate in dry mass, which was reduced significantly ($p < 0.05$) to 36.5% after 9 d pre-treatment by *T. reesei*. The yield of total structural carbohydrates in SH after *T. reesei* pre-treatment was reduced from 6.7 g/10 g dry substrate to 2.1 g/10 g dry substrate ($p < 0.05$) (Fig. 2a), which represents 68.6% reduction of the total structural carbohydrates. Glucans, xylan, arabinan, and mannan in SH were degraded significantly ($p < 0.05$) by 83.5%, 70.0%, 29.3%, and 50.6%, respectively. The *T. reesei* pre-treated WDGS/SH mixture showed significant ($p < 0.05$) reductions of glucan, xylan and arabinan by 64.5%, 27.0%, and 39.0%, respectively. When WDGS alone was pre-treated by *T. reesei*, the reduction ($p < 0.05$) of glucan, xylan, and arabinan were only 33.5%, 11.8%, and 25.6%, respectively. These observations indicate that the higher fiber degradation by *T. reesei* occurred when the substrates contained higher fiber content, which can induce production of carbohydrase by the fungal strain as reported by Li et al. (2017). As shown in Fig. S1 (Supplementary material), the morphology and appearance of *T. reesei* mycelium in SH was different compared with WDGS, with a more greenish color appearance in the pre-treated SH. Therefore, compared with WDGS, *T. reesei* was more efficient in the production of carbohydrase and degradation of fiber in SH.

3.1.2. *Effect of T. reesei pre-treatment on amino acids profile*

The amino acid concentration and yield in non-pre-treated SH and when *T. reesei* pre-treated SH, WDGS/SH mixture, and WDGS are shown in Fig. 3. Except for Cys, the concentrations of all the amino acid tested were enhanced in pre-treated SH, which was due to removal of structural carbohydrates (Fig. 2a) and no net change of total amino acid yield (Table 1). When considering the yield of each amino acid, a significant increase ($p < 0.05$) of only Thr was observed while the yields of other amino acid were either not significantly ($p > 0.05$) changed (Asp, Glu, Ser, Arg, Ala, Tyr, Val, Met, Phe, Ile, Leu, Lys, and Hyp) or reduced ($p < 0.05$) (His, Gly, and Cys). This indicates that *T. reesei* synthesized its own fungal protein that was rich in Thr while consumed His, Gly, and Cys in the substrate. It was reported from metabolic definition that some indispensable amino acid such as Met, Leu, Ile, Val, Phe can be synthesized from precursors that are structurally similar to these amino acids (keto-acids) and these precursors usually can be obtained from the diet. Dispensable amino acid (Ala, Asp, Glu, Ser) and conditionally essential amino acid (Arg, Cys, Gly, Tyr) can be synthesized from non-amino acid nitrogen compounds and carbon source such as α-keto acid. Therefore, the only essential amino acid from metabolic definition are Thr and Lys (Reeds, 2000). In pre-treated WDGS/SH and WDGS, significant ($p < 0.05$) reduction of amino acid yield was observed in Ser, His, Gly, Ala, Tyr, Cys, Val, Met, Phe, Ile, and Leu, however, no change was observed in Thr and Lys. Due to removal of total SC (Table 2), both Thr and Lys were significantly ($p < 0.05$) concentrated in pre-treated WDGS/SH by 31% and 24.7%, and in pre-treated WDGS by 19.9% and 28.4% (Fig. 3). Therefore, treating SH, WDGS/SH mixture and WDGS with *T. reesei* has the potential for enriching Thr and Lys that are essential for monogastric animal diets. However, as lesser amounts of the structural carbohydrates were degraded in WDGS/SH and WDGS than SH alone, the total amino acid content in WDGS/SH and WDGS was unlikely to be concentrated as shown in Fig. 3. In fact, the total amino acid concentration in WDGS/SH changed from 21.4 to 21.0% ($p > 0.05$), while in WDGS alone the total amino acid concentration was reduced from 23.7 to 22.9% ($p > 0.05$) (Table 2). This demonstrated that *T. reesei* is more likely to concentrate protein and amino acid in a fiber-rich substrate, and this ability may be limited when treating substrate with high concentration of protein. This can be explained by the switching from carbohydrase synthesis to protease synthesis in response to the change of inducer from fiber-rich substrate to protein-rich substrate (Ahamed and Vermette, 2008).

The total amino acid concentration in SH after treatment with *T. reesei* was significantly ($p < 0.05$) improved by 63.7% compared with the Ctrl treatment (Table 2). However, the total amino acid concentration had no significant difference ($p > 0.05$) in WDGS/SH mixture and WDGS after treated by *T. reesei*. Considering the weight loss during fungal treatment, there

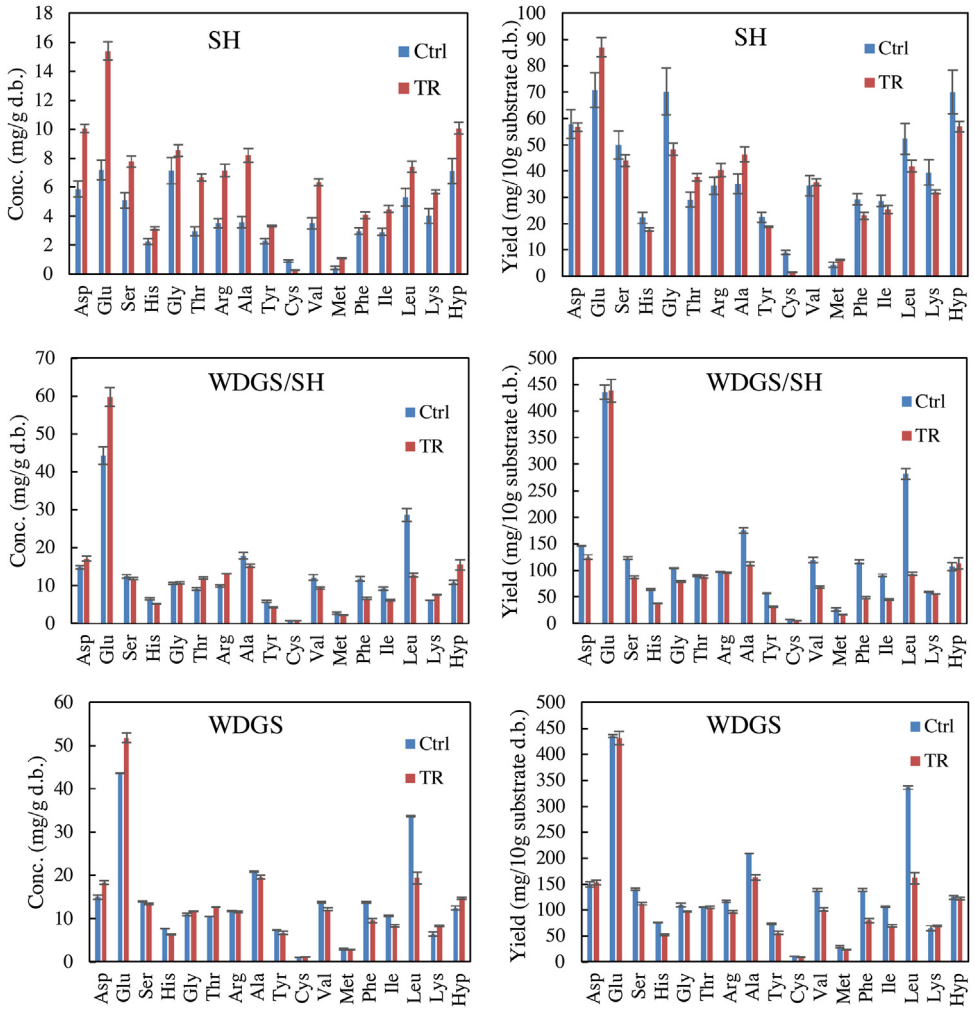

Fig. 3 – Concentration and yield of amino acids in non-pretreated (Ctrl) and *T. reesei* pretreated (TR) SH. The yield of amino acid reflects the weight change of the substrate before and after treatment. All values are means with error bars representing standard deviations of three replications.

Table 2 – Concentration and yield of total amino acids and structural carbohydrates, in *T. reesei* (TR) treated and non-treated (Ctrl) SH, WDGS/SH mixture and WDGS.

Component[a]	SH[b]		WDGS/SH		WDGS	
	Ctrl	TR	Ctrl	TR	Ctrl	TR
Total AA conc. (% d.b.)	6.7 ± 0.7	11.0 ± 0.4	21.3 ± 0.9	21.0 ± 0.5	23.6 ± 0.2	22.8 ± 0.3
Total AA yield (mg/10 g sub. d.b.)	659 ± 69	620 ± 25	2101 ± 42	1541 ± 42	2360 ± 32	1899 ± 36
Total SC conc. (% d.b.)	68.2 ± 1.0	36.5 ± 0.8	45.2 ± 2.4	33.3 ± 3.0	39.0 ± 0.2	37.1 ± 0.5
Total SC yield (mg/10 g sub. d.b.)	6707 ± 89	2064 ± 48	4454 ± 336	2443 ± 206	3891 ± 7.1	3084 ± 76

All the values are represented as mean ± standard deviation.

[a] AA: amino acids, SC: structural carbohydrates.

[b] SH: soybean hull, WDGS: wet distiller's grain with solubles.

was no significant change ($p > 0.05$) in the total amino acid yield in SH treated by *T. reesei* compared with Ctrl, while the total amino acid yield in the *T. reesei* treated WDGS/SH mixture and WDGS alone was reduced by 26.6 ($p < 0.05$) and 19.5% ($p < 0.05$), respectively. It was noted that 4643 mg out of the initial 6707 mg of structure carbohydrates in SH were degraded during pretreatment by *T. reesei* (Table 2), while only 116.2 mg of soluble sugar was produced. Because total amino acids yield did not change, it appears that an equivalent of 4525.8 mg of structural carbohydrates were converted to carbon dioxide, which accounted for around 46% of total dried substrate. Pre-treatment by *T. reesei* did not cause significant reduction ($p > 0.05$) of total amino acid yield in SH, which indicates that the enhanced total amino acid concentration in SH was exclusively due to the removal of structural carbohydrates. However, a significant reduction ($p < 0.05$) of total amino acid yield by 560 and 461 mg/10 g dry substrate was found in *T. reesei* treated WDGS/SH mixture and WDGS, respectively. Because of the reduction ($p < 0.05$) of total structural carbohydrate yield in WDGS/SH mixture and WDGS (by 2011 and 807 mg/10 g dry substrate, respectively), the total amino acid concentratin of both WDGS/SH and WDGS did not change after *T. reesei* pre-treatment. *T. reesei* has ability to produce both carbohydrase (cellulase and xylanase) and

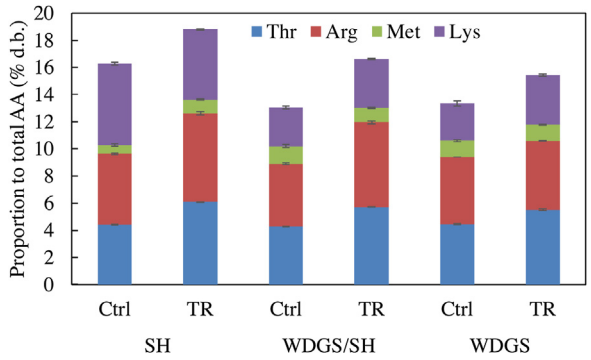

Fig. 4 – Proportion of four essential amino acids (Thr, threonine; Arg, arginine; Met, methionine; Lys, lysine) to total amino acids in *T. reesei* pre-treated (TR) and non-pre-treated (Ctrl) SH, WDGS/SH mixture and WDGS. All values are means with error bars representing standard deviations of three replications.

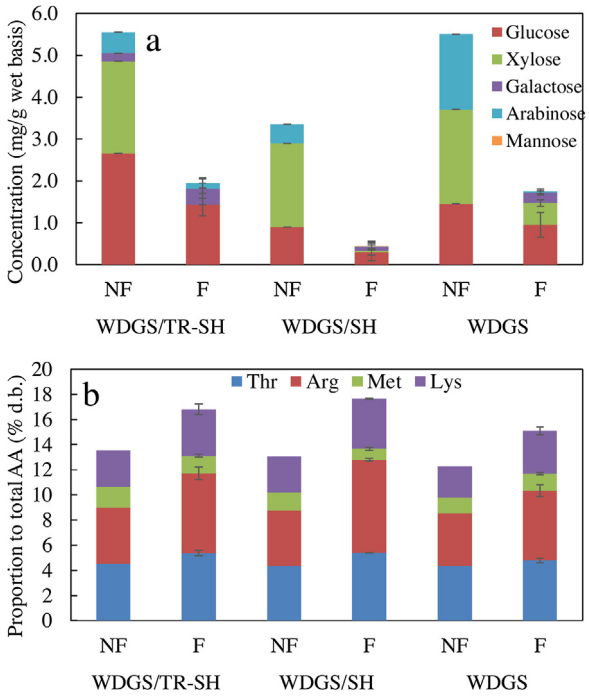

Fig. 5 – Reducing sugar concentration (a) and proportion of key amino acids (Thr, Arg, Met and Lys) to total amino acids (b) of *Aspergillus oryzae* fermented (F) and non-fermented (NF) WDGS mixing with TR-treated soybean hull (WDGS/TR-SH), WDGS mixing with soybean hull (WDGS/SH) and WDGS only (WDGS). All values are means with error bars representing standard deviations of three replications.

protease, and the production of which depends on the substrate composition (Li et al., 2017). *T. reesei* would be induced to synthesize carbohydrase in fiber-rich substrate such as SH alone to hydrolyze cellulose and hemicellulose into metabolizble sugars (monosaccharides) (Fig. 1b) and carbon dioxides. When the proportion of protein in substrate increased as in WDGS/SH mixture and WDGS alone, the expression of protease could be induced in *T. reesei* to hydrolyze protein into metabolizable amino acid for fungal growth and energy need. Moreover, the refractory structural carbohydrate reduction in WDGS could also be due to the complex xylan backbone in WDGS, which is indicated as arabinan/xylan ratio (Jaworski et al., 2015). The arabinan/xylan of WDGS and WDGS/SH was 0.86 and 0.84, respectively, higher than it in SH (0.69), which indicates more difficulty for *T. reesei* carbohydrase in degrading fiber in WDGS and WDGS/SH than in SH alone.

The balance of amino acid is critical for swine diet. A study had suggested that pig strongly preferred diets with balanced amino acid than diets with excessive amino acid (Edmonds et al., 1987). Moreover, diets with excessive of Thr, Arg, or Lys are more preferred by pigs than the diets with excessive of Met or Trp. Diets of imbalanced amino acid can be toxic and cause reduction of feed intake and impaired growth of pigs. Among these amino acid, Met and Cys are considered the most toxic amino acid, while Thr is the least toxic (Baker, 2006). Fortunately, the proportions of Thr and Arg were improved in all pre-treated substrate (SH, WDGS/SH and WDGS) by *T. reesei* (Fig. 4). The proportion of Lys was improved in substrate with WDGS (WDGS/SH and WDGS). This indicated that the pre-treated feed contributed to the balance of amino acid. It was found that the proportion of Asp, Glu, Gly and Ala were also increased after pre-treatment (Table S1, Supplementary material). However, these are non-essential amino acid and generally are not deleterious.

Amino acid profiles in the pre-treated SH, WDGS/SH and WDGS by *T. reesei* were contributed by both the substrate and the fungi biomass. The proportion of Asp, Glu, Thr, and Arg increased after pre-treatment of *T. reesei* regardless of substrate type. However, the ratio of Ala, Val, and Met to total amino acid were increased only in pre-treated SH, while Gly, Lys and Hyp were increased only in pre-treated WDGS/SH and WDGS. This indicated that *T. reesei* adapted its metabolisms and protein synthesis to better colonize substrates of different composition as observed from the different fungal appearance in Fig. S1 (Supplementary material). The improved proportion of Thr and Arg as two preferred amino acid by swine were exclusively originated from the fungal biomass pre-treating all three substrates. Therefore, it can be seen that pre-treating SH, WDGS/SH mixture and WDGS with *T. reesei* has economic value because it can strengthen the amino acid profile of the substrates with higher proportion of amino acid that have the lease toxic potential, thus improving the feeding value of the feedstock for swine nutrition.

3.2. *Fermentation of WDGS and SH by A. oryzae*

3.2.1. *Utilization of carbohydrates and improvement of key amino acids profile*

The concentration of each reducing sugar in the substrate mixtures before and after fermentation by *A. oryzae* is shown in Fig. 5a. Compared with WDGS/SH mixture, WDGS mixed with *T. reesei* pre-treated SH (WDGS/TR-SH) provides more glucose, xylose and galactose, which were generated during breakdown of cellulose and hemicellulose in SH by the *T. reesei* pretreatment (Figs. 1 and 2). Non-pre-treated WDGS/SH and WDGS alone, contained about 1.0–1.5 mg of glucose and 2.0 mg of xylose. These reducing sugars in WDGS could be from the unfermented sugar by yeast during ethanol fermentation. Lower concentration of glucose and arabinose, while no xylose, were observed in *A. oryzae* fermented substrates compared to non-fermented substrates. This demonstrates that xylose and arabinose were mostly or completely consumed by *A. oryzae* while glucose was partially utilized. The results were in line with a previous study showing that arabinoxylan can be synergistically degraded by xylanase, β-xylosidase,

and α-L-arabinofuranosidase from *A. oryzae*, which was known as soy source koji mold (Hashimoto and Nakata, 2003). In addition, galactose was produced in small amounts in all *A. oryzae* fermented substrates, due to the ability of *A. oryzae* in production of β-galactosidase which played roles in synthesis of galactooligosaccharides (GOS), a type of prebiotics (Zhu et al., 2020). Generally, diets rich in dietary fiber (non-starch polysaccharides, or cellulose and hemicellulose) have lower value for monogastric animals because of no digestive enzymes in these animals that can degrade the dietary fiber (Agyekum and Nyachoti, 2017). However, with fungal fermentation, the non-starch polysaccharides in fiber-rich substrates could be degraded into oligosaccharides which are readily digested by gut bacteria in monogastric animals such as swine, and therefore provide nutrients for the host and adjust the gastrointestinal microbiota (Holscher, 2017). Therefore, fermenting WDGS and SH or their mixture by *A. oryzae* have the potential of reducing non-starch polysaccharides and producing fermentable sugars or prebiotics which are beneficial for monogastric animals.

The proportions the four key amino acids together increased from between 12–14% to between 15–18% in all three substrate mixtures after fermentation by *A. oryzae* (Fig. 5b). Among them, the proportion of Thr, Arg and Lys each was improved while the proportion of Met was declined in the substrates after *A. oryzae* fermentation. Met had been considered as the most toxic amino acid if fed excessively in swine diet (Baker, 2006). Thr, Lys and Arg were evaluated to be preferred amino acid by swine in terms of feed intake and body weight gain than Met and Trp, if provided with excessive amount in the diet (Edmonds et al., 1987). Therefore, feed fermentation by *A. oryzae* would provide the feeding ingredients with better amino acid profile for swine nutrition. The total amino acid concentration of the three treatments with and without fermentation of *A. oryzae* is shown in Table S2 (Supplementary material). Because *A. oryzae* is an efficient protease producer (Chutmanop et al., 2008), it is understandable that total amino acids content in the substrates were reduced by 14.2%, 29.3% and 2%, respectively, in fermented WDGS/TR-SH, WDGS/SH, and WDGS alone after fermentation with *A. oryzae*.

3.2.2. *Effects of A. oryzae fermentation on phytate, lipid and in vitro dry matter digestibility*

Phytate is difficult to be digested by swine and poultry due to lack of phytase synthesis capacity in their gastrointestinal tracts, although it can be partially digested by some phytase-producing gut bacteria. Therefore, extrageneous phytase is usually added in diet to improve utilization of dietary components (Almeida and Stein, 2012). *A. oryzae* has the ability to degrade phytate during fermentation as shown in Fig. 6a. In dry grind ethanol production, phytate becomes concentrated in WDGS. About 11.5 mg/g (d.b.) of phytate was observed in WDGS. Its concentration reduced to 9.7 and 7.7 mg/g (d.b.) after mixing with pre-treated SH and SH which have low phytate content. After fermentation with *A. oryzae*, phytate levels in WDGS/TR-SH and WDGS were reduced by 74.6% and 71.4%, respectively, while by 53% in WDGS/SH mixture. The reduction of phytate was due to hydrolysis by phytase which was produced by *A. oryzae* during fermentation and a higher reduction of phytate occurred when the substrate contains higher concentration of phytate. The remaining phytate concentration after *A. oryzae* in three different substrates did not show significant difference ($p > 0.05$).

Lipids as highly digestible energy source plays important role in monogastric animal feed quality. As shown in Fig. 5b, total lipid content in WDGS can account for 17.5% of total dry mass. After mixing with SH or pre-treated SH (containing negligible lipid), the total lipid content of the mixture decreased to 13–14% of total dry mass. Fermentation of WDGS/TR-SH, WDGS/SH, and WDGS by *A. oryzae* resulted in lipid reductions of 8.4% ($p > 0.05$), 31.5 and 19.6%, respectively. WDGS/TR-SH preserved lipid from being consumed by *A. oryzae*, thus retained energy compounds in the fermented feed. The consumption of the total lipids in substrates by *A. oryzae* as carbon and energy source could be used for certain fungal metabolisms. The function of *T. reesei* treated SH in preventing lipid utilization by *A. oryzae* is not known based on current knowledge.

The total structural carbohydrates was higher in treatment WDGS/SH than it in WDGS/TR-SH and WDGS (Fig. 6c). However, the *in vitro* dry matter digestibility was lower in treatment WDGS/SH than it in WDGS/TR-SH and WDGS (Fig. 5d). It was reported that *in vitro* dry matter digestibility has strong negative correlation with non-starch polysaccharides (Jaworski et al., 2015). This was due to that higher content of cellulose and hemicellulose in WDGS/SH cannot be digested by pepsin and pancreatin during *in vitro* digestion. It was noted that all the three substrate mixtures after fermentation by *A. oryzae* show a lower *in vitro* dry matter digestibility than their non-fermented substrates. This could be due to lower total amino acid concentration (Table S2, Supplementary material) and lower total lipids (Fig. 6b) in *A. oryzae* fermented substrates than non-fermented substrate. Lower total amino acid indicates that the protein content was lower in fermented substrate, therefore, leading to less available protein for peptin hydrolysis during *in vitro* digestion. In addition, lower total lipid content in fermented substrate indicates less available lipids for pancreatin (containing lipase) hydrolysis during *in vitro* digestion. However, it was also noted that WDGS mixed with *T. reesei* pre-treated SH had higher *in vitro* dry matter digestibility (61.5%) than WDGS mixed with non-pre-treated SH (42.3%) and WDGS alone (56.3%). This higher *in vitro* dry matter digestibility in WDGS/TR-SH was partially attributed to concentrated total amino acid in *T. reesei* pre-treated SH.

3.2.3. *Effects of A. oryzae fermentation on in vitro digestibility of amino acids*

The *in vitro* digestibility of amino acid in the substrate mixture with and without fermentation by *A. oryzae* was shown in Table 3. Although amino acid in substrate were consumed by *A. oryzae* during fermentation, the resulting fermented feed when compared to non-fermented feed had higher digestibility of total amino acid. WDGS after fermentation showed 20% ($p < 0.05$) higher digestibility of total amino acid than its non-fermented control treatment. A slight improvement of total amino acid digestibility by 7.3% ($p > 0.05$) was found in fermented WDGS/SH mixture, while no improvement ($p > 0.05$) was observed in WDGS/TR-SH substrate. The improved total *in vitro* amino acid digestibility in fermented feed could be due to lower phytate content (Fig. 6a). Phytate can bind diatery protein electrostatically and form protein-phytate complexes (Kies et al., 2006) which are refractory to protein digestion by pepsin and also reduce ileal digestibility of amino acid (Selle and Ravindran, 2008). Therefore, degradation of phytate by *A. oryzae* could contribute to higher amino acid digestibility in the fermented feed by monogastric animals.

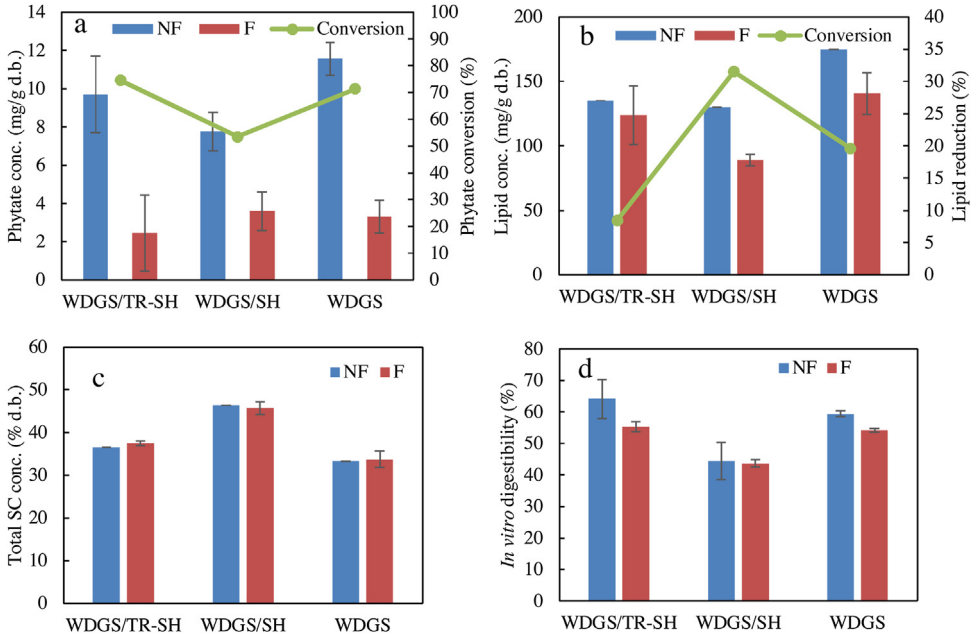

Fig. 6 – Concentration of phytate (a), total lipid (b), total structural carbohydrates (SC) concentration (c), and *in vitro* dry matter digestibility (d) in non-fermented (NF) and *Aspergillus oryzae* fermented (F) WDGS mixing with TR-pre-treated soybean hull (WDGS/TR-SH), WDGS with untreated soybean hull (WDGS/SH) and WDGS only (WDGS). All values are means with error bars representing standard deviations of three replications.

Table 3 – *In vitro* digestibility of amino acids in non-fermented (NF) and *A. oryzae* fermented (F) WDGS mixed with *T. reesei*-treated SH (WDGS/TR-SH), non-treated SH (WDGS/SH), and WDGS alone.

Amino acid	WDGS/TR-SH[a]		WDGS/SH		WDGS	
	NF	F	NF	F	NF	F
Asp	66.8 ± 1.8	72.8 ± 1.3	73.7 ± 0.6	85.4 ± 1.1	57.7 ± 0.5	74.4 ± 1.3
Glu	68.3 ± 3.7	74.5 ± 1.7	73.6 ± 0.3	83.2 ± 1.3	59.6 ± 0.2	74.0 ± 1.9
Ser	63.2 ± 3.2	64.1 ± 1.4	71.4 ± 0.4	76.8 ± 0.8	60.2 ± 0.5	71.3 ± 1.9
His	54.0 ± 3.8	56.9 ± 1.4	68.0 ± 1.5	73.2 ± 2.1	55.8 ± 1.4	67.7 ± 3.4
Gly	57.6 ± 2.3	57.7 ± 1.6	69.3 ± 1.0	73.4 ± 0.7	59.4 ± 0.3	69.7 ± 1.8
Thr	60.4 ± 3.1	66.0 ± 1.2	70.4 ± 0.7	78.8 ± 1.2	54.5 ± 0.6	70.6 ± 1.9
Arg	63.6 ± 1.5	47.0 ± 5.2	75.4 ± 1.7	68.6 ± 0.7	67.1 ± 1.1	62.0 ± 2.8
Ala	67.1 ± 3.0	57.6 ± 2.2	74.0 ± 0.8	72.4 ± 1.3	62.2 ± 0.1	66.7 ± 2.6
Tyr	65.3 ± 3.1	62.2 ± 2.2	75.1 ± 0.2	75.9 ± 1.5	64.6 ± 1.1	75.6 ± 3.4
Cys	50.1 ± 2.1	53.2 ± 1.7	65.2 ± 2.2	69.8 ± 0.7	47.5 ± 3.4	63.0 ± 3.4
Val	57.2 ± 2.9	60.0 ± 0.9	68.5 ± 0.6	73.7 ± 2.1	52.8 ± 0.6	66.9 ± 2.4
Met	70.4 ± 0.9	68.5 ± 2.7	77.9 ± 1.6	84.1 ± 3.1	61.9 ± 1.2	73.8 ± 4.7
Phe	66.5 ± 3.8	62.1 ± 1.5	73.6 ± 0.3	75.4 ± 1.5	61.4 ± 0.2	70.6 ± 2.4
Ile	62.9 ± 3.1	65.6 ± 1.2	71.1 ± 0.5	78.1 ± 1.3	57.2 ± 0.4	71.6 ± 2.0
Leu	64.3 ± 4.3	57.1 ± 2.0	71.7 ± 0.4	71.1 ± 2.3	57.2 ± 0.2	65.1 ± 3.6
Lys	60.7 ± 1.4	71.1 ± 1.1	73.2 ± 0.8	82.0 ± 1.6	62.2 ± 2.5	80.7 ± 1.7
Hyp	52.5 ± 2.1	63.9 ± 1.5	69.2 ± 2.4	82.5 ± 1.6	55.8 ± 1.3	77.6 ± 2.1
Total	63.6 ± 3.0	63.6 ± 0.7	72.2 ± 0.5	77.5 ± 1.2	59.0 ± 0.4	70.8 ± 2.0

All the values are represented as mean ± standard deviation.

[a] WDGS: wet distiller's grain with solubles, SH: soybean hull.

Overall, the amino acid digestibility in WDGS was affected by *A. oryzae* fermentation more than in WDGS/TR-SH and WDGS/SH mixtures. 14 out of 17 amino acid in WDGS had *in vitro* digestibility improved significantly ($p < 0.05$) after *A. oryzae* fermentation, higher than in WDGS/SH (9 out of 17 amino acid) and WDGS/TR-SH (5 out of 17 amino acid). However, except for Cys, Arg and Leu, all amino acid in non-fermented WDGS/SH had higher *in vitro* digestibility than non-fermented WDGS/TR-SH and WDGS. Moreover, most amino acid (Asp, Glu, Ser, Thr, Ala, Val, Met, Ile, and Leu) in fermented WDGS/SH mixture had significantly ($p < 0.05$) higher *in vitro* digestibility than fermented WDGS/TR-SH and WDGS. The digestibility of Thr and Lys in the fermented product in all treatments was significantly ($p < 0.05$) improved compared to their non-fermented control. Thr and Lys are considered as the only essential amino acid from metabolic definition and usually should be provided extrageneously if they are considered as limited in swine diet (Reeds, 2000). The improved *in vitro* digestibility of larger number of amino acid in WDGS indicates that *A. oryzae* fermentation is more effective in improving amino acid digestibility in WDGS than its mixture (WDGS/TR-SH and WDGS/SH). In addition, higher amino acid digestibility of both non-fermented and fermented WDGS/SH

than their corresponding substrates showed advantages of mixing WDGS with SH regardless of *A. oryzae* fermentation.

Due to limitations of certain nutrients (amino acid, dietary fiber, lipid, phosphorous, *etc.*), single feedstocks are commonly combined with other feedstocks, for example corn grain and soybean meal, to balance or enrich certain dietary components required by monogastric animals. Fermentation of these feedstocks harnesses the unique capabilities of microorganisms to modify the feedstocks by removing unfavorable components while improving favorable nutrients. Both *T. reesei* pre-treatment and *A. oryzae* fermentation of WDGS, SH or their mixtures could serve as effective approach of feed nutrition improvement for monogastric animals by reducing fiber content, concentrating amino acid, improving proportion of essential amino acid, reducing phytate, preserving total lipid, and improving dry matter and amino acid digestibility. Therefore, the combined use of fungal species should be considered in balancing the nutrients while minimizing negative effects of anti-nutritional factors on feed quality. In addition, because soybean hull, rich in fiber, has substantial loss of dry mass during *T. reesei* pre-treatment, the mass loss of SH or other fiber-rich feedstock used should be considered as an important factor in large scale application. Future research is still required as to select more desired fungal strains with lower proclivity for consuming amino acid, lipids, *etc.* which are important components as monogastric animal feed. In addition, better fermentation strategies, cost-efficient microbial or fungal nutrients (low-cost carbon and nitrogen source) may also need evaluation to improve microbial activity which further facilitates nutrient utilization of both in substrate and in microbial biomass and metabolites.

4. Conclusion

This study investigated the effects of two fungal strains *T. reesei* and *A. oryzae* on soybean hull, WDGS, and their mixtures. The pre-treated soybean hull by *T. reesei* had lower fiber content (by 69%) and concentrated amino acid (by 7–11%). The WDGS mixed with *T. reesei* pre-treated soybean hull after *A. oryzae* fermentation had lower content of phytate (by over 50%), higher lipid content and improved dry matter digestibility. In addition, *A. oryzae* fermented WDGS and its mixture with soybean hulls had higher digestibility of total amino acid including essential amino acid Thr and Lys. This study demonstrated the advantages of fungal pre-treatment and fermentation in improving feeding value of WDGS and soybean hull.

CRediT author statement

Xiao Sun: Investigation, Writing-original draft preparation, Writing-review and editing, Data curation, Methodology, Formal analysis. **Nongmaithem Debeni Devi:** Investigation, Methodology. **Jae-Cheol Jang:** Methodology. **Pedro E. Urriola, Douglas G. Tiffany, Gerald G. Shurson, Bo Hu:** Funding acquisition, Conceptualization, Supervision, Writing-review and editing.

Conflict of interest

The authors declare that there is no conflict of interest related to this manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fbp.2021.09.013.

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