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Nutrition upgrading of corn-ethanol co-product by fungal fermentation: Amino acids enrichment and anti-nutritional factors degradation

Xiao Sun^a, Douglas G. Tiffany^a, Pedro E. Urriola^b, Gerald G. Shurson^b,
Bo Hu^{a,*}

^a Department of Biosystems and Agricultural Engineering, University of Minnesota, 1390 Eckles Ave, St. Paul, MN 55108, USA

^b Department of Animal Science, University of Minnesota, St. Paul, MN 55108, USA

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ABSTRACT

Corn wet distiller's grain with solubles (WDGS) used as a feeding ingredient to monogastric animal diets is limited due to its imbalanced key amino acids, high fiber and potential existence of mycotoxins. Fermentation of WDGS by edible fungi has potential of upgrading the feedstock into nutritional and low-risk feeding ingredient for monogastric animals. In this study, four different fungi *Aspergillus oryzae*, *Rhizopus oryzae*, *Mucor indicus* and *Trichoderma reesei* were employed to ferment the mixture of WDGS and soybean hull at 75/25 ratio at 28 °C for 6–9 days. Urea at different concentration was supplied as additional nitrogen source. Results showed that, *M. indicus* improved total amino acids yield by 13.3% with over 95% consumption of the supplied urea at 1% N. Meanwhile, *T. reesei* degraded structural polysaccharides by 41% and concentrated amino acids by 19%. In addition, phytate was degraded by 53, 56, 31 and 20% with *A. oryzae*, *R. oryzae*, *M. indicus*, and *T. reesei*, respectively. Total aflatoxin and deoxynivalenol was detoxified via *T. reesei* by 52.8% and 92.9%, respectively, while zearalenone was detoxified via *M. indicus* by 89.2%. This study demonstrated a feasible way of producing nutritional-improved monogastric feeding ingredient from corn-ethanol plant.

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1. Introduction

Improving feeding value of agricultural-industry co-products for animal production has been considered beneficial to human health and nutrition, and meanwhile contribute to the fuel-energy-water (FEW) nexus of our planet. Corn (*Zea mays* L.) accounts for 94% of total feedstock used for ethanol production in U.S., and around 91% of the corn is converted via dry mill process to ethanol and co-products (mainly corn distiller dried grains with solubles, or DDGS.) (RFA, 2020). DDGS after a series of downstream processing are used as ingredients in animal feed with limited inclusion ratio, which can sometimes account for up to 25% of total revenue for certain corn ethanol facilities (Hill et al., 2006). Soybean provides nutrition globally both as whole grains, soy-

bean oil and soybean meal for human and animals (Jia et al., 2020). However, soybean hull (SH) as a co-product of soybean processing industry has lower value and has been commonly used as fiber source supplement for ruminant diet. The conversion of corn starch (accounts for approximately 70% of total mass) into ethanol during dry-milling process resulted in the DDGS having higher concentration of protein, non-starch polysaccharides (mainly cellulose and hemicellulose) and phytate, as compared to corn grain. Compared to soybean meal, however, proteins in DDGS contain lower concentration of key amino acids such as arginine (Arg), lysine (Lys), methionine (Met), and threonine (Thr), which were reported to be limiting amino acids in diets of swine, poultry and fish (Farkhoy et al., 2012; Liao et al., 2015; Lim et al., 2011). Imbalance of these amino acids in DDGS limited its inclusion

* Corresponding author at: University of Minnesota, Department of Bioproducts and Biosystems Engineering, 1390 Eckles Ave, Saint Paul, MN, 55108-6005, USA.

E-mail address: bhu@umn.edu (B. Hu).

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ratio in monogastric animal diet. Moreover, hemicellulose and phytate are non-digestible component to monogastric animals, resulting in increased manure production that causes management and environmental issues (Chen et al., 2013). Phytate (inositol polyphosphate) as a major storage form of phosphorous in WDGS is unavailable to monogastric animals because their endogenous mucosal phytase is unable to hydrolyze enough phytate (Humer et al., 2015). Moreover, some mineral nutrients such as calcium and zinc and dietary nutrients such as protein, lipid and starch could be chelated with phytate and become less available for the use of monogastric animals (Selle and Ravindran, 2008). One of the most safety concerns about DDGS is the presence of mycotoxins which are generated by pathogenic mold during inappropriate storage of corn grain and DDGS (Zhang and Caupert, 2012). Aflatoxins (produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*), zearalenone (ZEN) and deoxynivalenol (DON) (produced by *Fusarium* species) are the most toxic and prevalent mycotoxins that cause loss of animal and human life, loss of livestock, meat, milk and egg production, and loss of crops and feeds globally (Ji et al., 2016). Mycotoxins remaining in DDGS could significantly limit its inclusion ratio or simply be prohibited as feeding ingredient for certain animals (Zain, 2011). Therefore, increasing key amino acids, reducing non-starch polysaccharides in DDGS and reducing anti-nutritional factors including phytate and mycotoxins have potential to raise its feeding value for monogastric animals and increase benefits of corn-ethanol refineries. In addition, upgrading SH to a more nutritional feeding ingredient to swine, poultry and aquaculture life could promote its utilization in animal feeding market with lower cost for animal production, thus bringing potential revenues to the rural economy.

Biological processing is an environmental-friendly and economic route of upgrading animal feeding material (Ji et al., 2016). Fungi used during biological processing for feed production are generally regarded as safe (GRAS). *Rhizopus oryzae* (R. *oryzae*), *Mucor indicus* (M. *indicus*), *Aspergillus oryzae* (A. *oryzae*) and *Trichoderma reesei* (T. *reesei*) were commonly used fungal strains for production of human food or food-grade enzymes (Benabda et al., 2019; Hoa and Hung, 2013; Karimi and Zamani, 2013; Li et al., 2017). The enzymes produced from these fungal strains include carbohydrase (Ezeilo et al., 2020), proteinase (Li et al., 2017), phytase (Sun et al., 2021), which were proved as important enzymes for feed nutrition improvement. Apply of enzymes to corn-ethanol fermentation process had also been reported to improve protein, amino acids, fatty acids and minerals of the remaining DDGS (Fang et al., 2018). However, as cell factory, fungal strains do more than enzymes production which include converting low-value compounds into high valued ones, breakdown of indigestible component such as phytate, and detoxify undesirable compounds such as mycotoxins (Ji et al., 2016). Urea as a low-cost nitrogen source with high nitrogen content has long been used as a replacement of vegetable and animal protein source in ruminant diet due to the presence of a significant quantity and diversity of rumen ureolytic microorganisms in their gastrointestinal tracts (Chavez et al., 1966; Komegay et al., 1965; Matsumoto, 1955). In comparison, monogastric animals such as swine and poultry showed relatively low quantity and diversity of ureolytic bacteria which are unable to convert large amount of feeding urea into microbial protein (Patra and Aschenbach, 2018). Therefore, urea supplemented to fungal bioprocessing could have potential of contributing fungal biomass and metabolisms, therefore improving amino acids profile and reducing anti-nutritional factors of the fermented feed.

This study on improving nutritional value while degrading anti-nutritional compounds of co-products from corn-ethanol plant and soybean processing plant by biological processing with different fungal strains had never been reported. In this study, WDGS mixing with SH was used as substrate for fermentation by R. *oryzae*, M. *indicus*, A. *oryzae*, and T. *reesei* with supplementation of different concentration of urea. Amino acids, urea, non-starch or structural polysaccharides, and dry weight changes were monitored before and after fermentation to evaluate the capability of amino acids enrichment, fiber degradation and urea utilization by the different fungal strains. In addition, phytate, total aflatoxins, zearalenone, and deoxynivalenol were measured

before and after fermentation to evaluate detoxification ability by each fungus.

2. Materials and methods

2.1. Feedstocks

WDGS was supplied by a dry-milling corn (*Zea mays* L.) ethanol plant (Absolute Energy, St. Ansgar, IA) and was stored frozen at -20 °C upon received. Soybean hull (SH) were purchased from Republic Mills, Inc., Okolona, OH, and was stored at dry and cool area upon receipt. SH are light and bulky with less than 10% moisture content. When mixing with WDGS (around 50% moisture), the WDGS/SH mixture could provide organic substrates and support for fungal strains with appropriate moisture content, large specific surface area for colonization, and large inner space for ventilation. WDGS and SH from suppliers had particle size of less than 2 mm and was directly used for substrate preparation without milling.

2.2. Fungal strains preparation

T. *reesei* (ATCC 28217), M. *indicus* (ATCC 24905), and A. *oryzae* (ATCC 1011) were purchased from American Type Culture Collection (ATCC). R. *oryzae* was isolated from the seeds of evening primrose (*Oenothera biennis*), a species of flowering plant in the family Onagraceae which is native to eastern and central North America. Potato dextrose agar (PDA) medium (Actero™, FoodChek Systems Inc., Calgary, Alberta, Canada) containing (L): 4.0 g of potato infusion, 20.0 g of dextrose, 15.0 g of agar, with pH of 5.6 were sterilized (121°C for 15 min) and prepared in petri-dish to maintain T. *reesei* for 7 days and R. *oryzae*, M. *indicus* and A. *oryzae* for 4 days before transferring to new PDA medium or being used for inoculum preparation. This was to maintain the activity of each fungal strain to develop consistent mycelia and spores. To obtain inoculum with active growth rate to minimize lag phase after fermentation, sub-culturing of each strain was performed in sterilized potato dextrose broth (PDB) medium (Difco™, BD Diagnostics, Fracklin Lakes, NJ, USA) containing (L): 4.0 g of potato starch, 20 g of dextrose, and with pH of 5.1. To do sub-culturing, five pieces of the fully grown fungal mycelia (each piece around 0.5 × 0.5 square cm) of each strain in PDA medium were cut with sterilized flat-top spatula (Supplementary material Fig. S1) and transferred to 100 mL of freshly prepared PDB medium in each of 250 mL Erlenmeyer flasks. The initial number of spores in the 100 mL of PDB medium for each strain was counted using Cellometer Auto×4 Cell Counter (Nexcelom Bioscience LLC, Lawrence, MA, USA) and the count of spores for R. *oryzae*, M. *indicus*, T. *reesei* and A. *oryzae* were approximately 1.61×10^8 , 8.27×10^7 , 1.45×10^7 , and 7.79×10^6 CFU, respectively. The sub-cultures for all strains were shaken at 150 rpm in an orbital shaker for 3 days at 28 °C for a good development of pelletized mycelia (size between 1 to 3 mm) that were used as inoculum for the subsequent solid-state fermentation.

2.3. Solid-state fermentation of WDGS/SH mixture by different fungi with supplementation of urea

The use of urea in monogastric animals was less efficient than ruminant due to lack of efficient gut bacteria. In vitro synthesis of protein or amino acids from urea by fungal strains could provide chance of effective use of urea for monogastric animals. The effect of urea supplement on amino acids

accumulation, fiber degradation, and anti-nutritional factors reduction during fermentation of WDGS/SH mixture was evaluated with four different fungal strains, *A. oryzae*, *R. oryzae*, *M. indicus*, and *T. reesei*. The total dry weight of substrate mixture in each 250 mL Erlenmeyer flask was kept at 10 g which included 7.5 g dry mass of WDGS and 2.5 g dry mass of SH (based on preliminary study). The initial moisture content of the substrate was kept at 70% (w/w) (Barnharst et al., 2021) which was contributed by the moisture of WDGS (50% w/w), SH (9% w/w), inoculum (5 mL) and additional deionized water (for treatment without urea) or prepared urea solution (for treatments with urea supplement). Therefore, for each flask, fifteen grams of WDGS (wet basis) and 2.75 g of SH (wet basis) were mixed followed by addition of 10.58 mL of DI water or urea solution. The 70% (w/w) initial moisture content was achieved after inoculating with 5 mL of sub-cultured fungal strain.

Urea ($\text{CH}_4\text{N}_2\text{O}$) contains 47% of nitrogen on mass basis and was added based on nitrogen/substrate ratio (on dry mass basis). Nitrogen/substrate ratio of 1% (N-1%), 2.5% (N-2.5%), 5% (N-5%) and control without adding urea (none) were tested for *A. oryzae*, *M. indicus*, *R. oryzae*, and *T. reesei* during fermentation of WDGS/SH mixture. Urea for each nitrogen/substrate ratio was measured and dissolved in DI water to make different concentration of urea solution (N-1%: 20.11 g/L; N-2.5%: 50.28 g/L; N-5%: 100.55 g/L), which was then added to each corresponding treatment at the volume mentioned above. DI water of the same volume was added to treatments without urea supplement. Each flask with prepared substrate was plugged with foam stopper (JAECCE Industries, Inc., NY, USA) to allow air exchange while preventing possible contamination during fermentation. Aluminum foil was used to cover the stopper of each flask before sterilization (121 °C for 20 min) to prevent moisture loss during stay in autoclave.

The sterilized substrate in each flask was then inoculated aseptically with 5 mL of the sub-culture of each fungal strain. The control treatment (Ctrl) without fermentation was inoculated with the same volume of sterilized (121 °C for 30 min) sub-culture of corresponding fungal strain (deactivated strain). After inoculation, the substrate in each flask was hand-shaken briefly to homogenize the fungal mycelia and spores. Incubation for non-inoculated and *A. oryzae*, *M. indicus*, and *R. oryzae* inoculated substrate was conducted statically for 6 days while *T. reesei* for 9 days (due to relatively slow growth compared to other fungal strains). Incubation of all treatments were performed in a 28 °C incubator with continuous air circulation by an installed fan to provide consistent air exchange for all the flasks. After fermentation, the flasks with fermented and non-fermented (Ctrl) substrate were dried in a 60 °C drying oven for 48 h to have a moisture content below 10% (w/w). The dried sample was then milled into fine particles with coffee grinder and stored in –20 °C freezer for further analysis. Weight of each empty flask before substrate preparation and of the same flask containing dried sample were measured to monitor the weight loss of the substrate during fermentation. Each treatment was performed in triplicates.

2.4. Analytical methods

2.4.1. Amino acids, structural polysaccharides, soluble sugar and urea analysis

The dried and ground solid samples were hydrolyzed to breakdown protein into amino acids. Each sample (50 mg) was mixed with 1.0 mL of 6 M HCl solution and hydrolyzed at 110 °C for 24 h in a 2 mL sealed centrifuge tube. The headspace of each

tube was purged with pure nitrogen before hydrolysis to avoid oxidation and degradation of certain amino acids (methionine and cysteine). The hydrolyzed samples were diluted and filtered through 0.22 µm Nylon filter before quantification. The analysis was performed in high performance liquid chromatography (HPLC, 1200 Infinity series, Agilent Technology, Santa Clara, CA, USA) equipped with ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 3.5 µm) (Agilent Technologies, Inc.) and Diode array detector (DAD) using UV light source. The amino acids in each sample and standards were derivatized by ortho-phthalaldehyde (OPA) and 9-fluorenyl-methyl chloroformate (FMOC) (Agilent Technology, Santa Clara, CA, USA) in place by HPLC auto sampler (G1329A, Agilent Technologies, Inc.) before injection (Henderson and Brooks, 2010). Two mobile phases were used. Mobile phase A contains (/L): 10 mmol Na_2HPO_4 , 10 mmol $\text{Na}_2\text{B}_4\text{O}_7$, 5 mmol NaN_3 , pH 8.2 (adjusted with concentrated HCl). Mobile phase B contains (/L): 450 mL acetonitrile, 450 mL methanol, 100 mL ultra-pure water. Amino acids standards were prepared and calibrated based on procedures described (Henderson and Brooks, 2010).

Structural polysaccharides (glucan, xylan, arabinan, galactan, mannan) of the samples before and after fermentation were determined with two-step acid hydrolysis method based on NREL protocol (Sluiter et al., 2008). The hydrolyzed samples containing monomeric sugars (glucose, xylose, arabinose, galactose, and mannose) after filtration with 0.22 µm Nylon filter were determined with HPLC equipped with Biorad Aminex HPX-87P analytical column (300 × 7.8 mm) and refractive index detector (RID). Ultra-pure water was used as mobile phase at flow rate of 0.6 mL/min. The polymeric sugars were converted from their corresponding monomeric sugars using an anhydro correction of 0.88 (132/150) for C5 sugars (xylose, arabinose) and a correction of 0.9 (162/180) for C6 sugars (glucose, galactose, mannose).

Grinded dry samples were soaked with DI water at a ratio of 1:10 (w/v) at 4 °C overnight. The mixture was then centrifuged at 13,000 for 10 min to settle down the solids while the supernatant was collected for analysis of urea, pH, and soluble sugar. For analysis of urea and soluble sugar, the supernatant was further diluted as required to allow the detected value within calibration range. The concentration of urea was determined based on diacetyl monoxime method (Rosenthal, 1955). Briefly, urea reacts directly with diacetyl monoxime under strong acidic conditions to give a yellow condensation product. The reaction was intensified to red colour by the presence of ferric ions and thiosemicarbazide (stabilise colour) (Mahesh et al., 2019). The concentration of soluble sugars containing monosaccharides (glucose, xylose, galactose, arabinose, mannose) and cellobiose were determined using HPLC after filtrating through 0.22 µm Nylon member filter. The HPLC condition was the same as it for analyzing structural polysaccharides. pH for the supernatant of each sample was determined directly with electronic pH meter.

2.4.2. Analysis of phytic acid and mycotoxins

Phytic acid concentrations in non-fermented (Ctrl) and fermented treatments by *A. oryzae* (AO), *R. oryzae* (RO), *M. indicus* (MI) and *T. reesei* (TR) were determined using Phytic acid assay kit (Megazyme). The concentrations of total aflatoxin, deoxynivalenol (DON) and zearalenone (ZEN) for each treatment was determined using their ELISA assay kits (BioVision). The lower detection limit for total aflatoxin is 0.2 ppb (part per billion), for DON is 150 ppb, and for ZEN is 6 ppb. The 96-well microplate for each mycotoxin assay was prepared by

the manufacturer and was directly used following the instruction in each kit. Microplate Bio-kinetics reader (EL340, Bio-Tek Instruments, Winooski, VT, USA) was used to determine the absorbance of each well on the microplate at 450, 405, or 630 nm wavelength.

2.5. Calculation and statistical analysis

The term Yield was used to define the quantity of analyte considering the substrate mass loss during solid-state fermentation. The data of yield was based on initial dry mass of substrate (10 g). The results related to amino acids, urea, structural polysaccharides, soluble sugars, phytate and mycotoxins were expressed after calculation using one or more of Eqs. (1)–(3) shown as below:

$$\text{Conc. of } i \text{ (mg/g d.b.)} = \frac{\text{Mass of } i \text{ (mg)}}{\text{Dry mass of solid sample (g)}} \quad (1)$$

$$\text{Yield of } i \text{ (mg per initial substrate)} = \text{Conc. of } i \text{ (mg/g d.b.)} \times \text{Dry mass of solid sample (g)} \quad (2)$$

$$\begin{aligned} \text{Conversion of } i \text{ (\%)} \\ = \frac{\text{Initial mass of } i \text{ (g)} - \text{remaining mass of } i \text{ (g)}}{\text{Initial mass of } i \text{ (g)}} \times 100 \end{aligned} \quad (3)$$

where: d.b., dry basis; Conc., concentration; i: analyte (amino acid, urea, structural polysaccharide, soluble sugar, phytate, total aflatoxin, ZEN or DON).

The statistical analysis was performed with Tukey's multiple comparison of means at 95% confidence interval using JMP Pro 14.0.0 (SAS Institute Inc., Cary, NC, USA). The statistical analysis was used to determine pair-wised statistical differences ($p < 0.05$) of amino acids, urea, structural polysaccharides, soluble sugars, phytate and mycotoxins. Data in figures and tables were presented as mean of three replications with standard deviation shown as error bar in figures.

3. Results and discussion

3.1. Effects of urea on amino acids accumulation by different fungal strains

The pH, total AA concentration, and AA yield in the substrate after fermentation with different fungal strains under different urea level were shown in Fig. 1A. pH in non-fermented substrate (Ctrl) showed increased value from 4.5 in substrate without urea to 5.6 in substrates with increased urea supply, which could be due to the partial decomposition of urea into ammonia (NH_3) (Yim et al., 2004) under high temperature during autoclave. Urea decomposition can also be witnessed by the reduced concentration of urea detected in the non-fermented substrate (Ctrl) (Fig. 1B) as compared to the initial urea supplied. It was noticed that around 45%, 31%, and 24% reduction of urea, and corresponding 0.81, 1.07, and 1.07 unit increase of pH were occurred, respectively, for the sterilized substrate with supply of 1% N, 2.5% N, and 5.0% N of urea. However, compared to bacteria, fungal growth was less strongly affected by pH between the range of 4.0–8.3 (Rousk et al., 2010). Therefore, no pH adjustment was conducted before inocula-

tion. Compared to substrate with urea, the substrate without urea after fermentation showed the highest pH increase from 4.5 to between 5.5 and 6.0 by all fungal strains. Moreover, the fermented substrate with or without urea by each fungal strain had a final pH between 5.3 and 6.1, which may not be inhibitive to the fungal growth. Therefore, the initial high concentration of urea or its decomposed by-product (NH_3) could be the reason for fungal growth inhibition.

Total AA concentration and yield in non-fermented substrate (Ctrl) did not show significant change ($p < 0.05$) at different urea concentration, suggesting no abiotic interactions between urea and AA in substrate. Without urea addition, the total AA yield in substrate after fermentation was reduced by 40.6% with *A. oryzae*, 23.8% with *R. oryzae*, 9.2% (not significant, $p > 0.05$) with *M. indicus*, and 29.8% with *T. reesei*. Production of protease that can breakdown protein into peptides and amino acids for metabolisms of organisms had been widely reported using *A. oryzae* (Zhao et al., 2019), *R. oryzae* (Benabda et al., 2019), and *T. reesei* (Sun et al., 2021). However, protease production from *M. indicus* was rarely reported (Karimi and Zamani, 2013). No consumption of AA in *M. indicus* treated substrate could be due to lack of protease synthesized by *M. indicus*. This could render *M. indicus* ability to accumulate cell biomass with rich AA without consuming existing protein in substrates, thus improving AA profile of the final product.

When urea at 1% N was added to the substrate, all strains showed improvement of total AA compared to the treatment without urea addition. The total AA concentration after fermentation improved by 10.1% with *A. oryzae*, 14.0% with *R. oryzae*, 18.6% with *M. indicus*, and 23.7% with *T. reesei*, as compared to treatment without urea. Moreover, the total AA concentration in *R. oryzae*, *M. indicus*, and *T. reesei* was 5.9%, 25.1%, and 18.7% higher, respectively, than Ctrl treatment. *M. indicus* showed an improved AA yield by 13.3%, equivalent to 288 mg net AA production out of 2160 mg AA in substrate (Ctrl). From Fig. 1B, around 112 mg of urea was consumed by *M. indicus* in the substrate supplied with 1% N of urea. This indicates that *M. indicus* could use 1 g of urea to produce 2.57 g of total AA, assuming no other nitrogen compound was produced during fermentation. It was also noted that more than 80% of urea was utilized by *A. oryzae*, *R. oryzae*, and *T. reesei*, but no net AA was synthesized, which could be due to that the urea was used as nitrogen source for metabolisms (Navarathna et al., 2010) instead of biomass accumulation. With urea addition increased to 2.5% N, the total AA concentration increased accordingly in *A. oryzae* and *R. oryzae*, while a slight decrease or non-change was observed for *T. reesei* and *M. indicus*. The same trend also applied to total AA yield in treatments with *A. oryzae*, *R. oryzae* and *M. indicus*. For *T. reesei*, the higher total AA yield in 2.5% N treatment than 1% N treatment indicates less consumption of substrate AA and more utilization of urea as nitrogen source (104.6 mg of urea used in 1% N compared to 253.7 mg of urea used in 2.5% N, per initial substrate). Except for *R. oryzae*, all the three fungal strains consumed more urea in the substrate with 2.5% N supply than it with 1% N (Table 1). Addition of urea to 5% N in substrate further promoted total AA concentration in *A. oryzae* and *R. oryzae*, while no much increase was found in *M. indicus*. With *T. reesei*, neither increased concentration nor additional yield of total AA was observed compared to Ctrl, indicating an occurrence of severe inhibition of *T. reesei* growth. Under this condition, *A. oryzae*, *R. oryzae*, *M. indicus*, and *T. reesei* still consumed 113.6, 72.3, 345.1, and 50.9 mg of urea per initial substrate, respec-

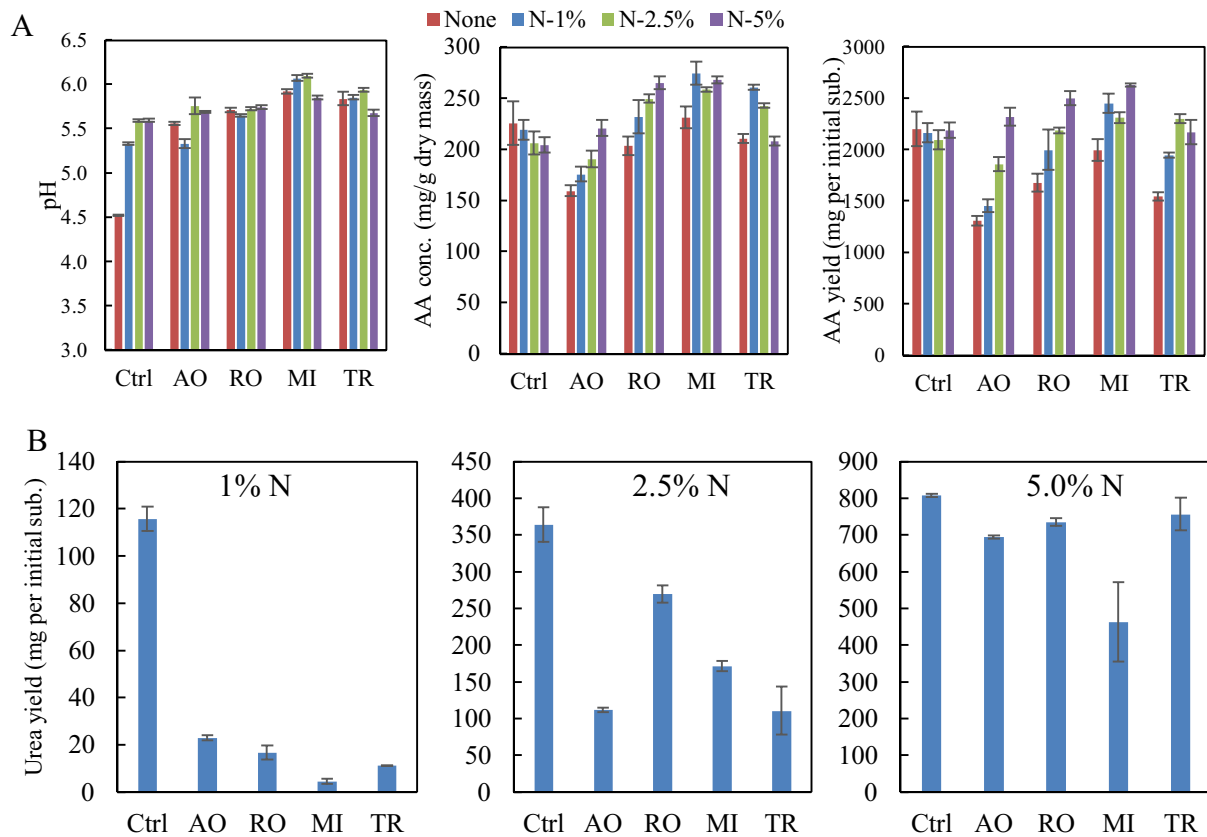


Fig. 1 – Amino acids concentration and yield (A) and urea yield (B) in WDGS/SH (75/25) mixture without (Ctrl) and with fermentation by *A. oryzae* (AO), *R. oryzae* (RO), *M. indicus* (MI), and *T. reesei* (TR). All values are means with error bars representing standard deviations of three replications.

Table 1 – Urea conversion efficiency and consumption by each fungal strain.

	Conversion			Total urea consumption ^b		
	%			mg per initial substrate		
Fungal strain ^a	N-1%	N-2.5%	N-5%	N-1%	N-2.5%	N-5%
AO	80.14	69.29	14.05	92.85	252.65	113.55
RO	85.50	25.97	8.95	99.06	94.69	72.31
MI	95.98	52.95	42.70	111.21	193.06	345.10
TR	90.29	69.59	6.30	104.61	253.73	50.88

^a AO: *A. oryzae* RO: *R. oryzae*, MI: *M. indicus*, TR: *T. reesei*.

^b Total urea consumption was calculated by subtracting urea yield of treatment AO, RO, MI or TR from that of Ctrl.

tively (Table 1). The total AA yield in *R. oryzae* and *M. indicus* was still significantly higher ($p < 0.05$) than it in Ctrl.

It was noticed from Table 1 that *M. indicus* could utilize up to 345.1 mg urea when around 808 mg of urea (Fig. 1B, 5% N treatment) was present in initial substrate (dry basis). In addition, *A. oryzae* and *T. reesei* consumed the highest 252.6 and 253.7 mg urea per initial substrate, respectively, when around 365 mg of urea (2.5% N treatment) was present in substrate. *R. oryzae* consumed 99.1 mg urea per initial substrate when 115.9 mg urea was present (1.0% N treatment), and its consumption reduced when urea supply increased (Table 1). However, from economic point of view, supplement of 1% N equivalent of urea to substrate resulted in high urea conversion efficiency (greater than 80%) by all four fungal strains (Table 1), minimizing the unnecessary lost of urea. Urea is not easily digested by the monogastric animals due to the lack of urease producing bacteria in their gastric intestinal tract (Patra and Aschenbach, 2018). However, the possible urease activity in the fungal strains studied allows utilization of urea as nitro-

gen source for the synthesis of amino acids, thus improving quality and quantity of protein in the fermented feed. Considering the total net synthesized AA under 1% N supply with urea, *M. indicus* would be the strain of choice for the purpose of protein and amino acids enrichment.

Improving essential AA in WDGS/SH mixture could increase its nutritional value thus promoting its inclusion rate in monogastric animal diet. Seven essential AA, threonine (Thr), arginine (Arg), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), and lysine (Lys) under different urea supply levels after fermentation by each of four fungal strains were compared in Table 2. Similar to total AA concentration and yield in Fig. 1A, total essential AA was declined in substrate without urea supply (None) after fermentation by each fungus as compared to Ctrl. However, Arg was increased after fermentation by *A. oryzae*, *R. oryzae* and *M. indicus*, while Lys was improved only in *M. indicus*. This indicates that essential AA could not be enriched at the condition without additional supply of nitrogen source. In substrate with supply of 1% N

Table 2 – Yield and concentration of key amino acids in non-fermented (Ctrl) and fermented WDGS/SH (75/25) mixture with increased urea supplement.

Urea	Fungi ^a	Thr ^b Yield (mg per initial substrate)	Arg	Val	Met	Ile	Leu	Lys	Total key AA	Thr Conc. (mg/g d.b.)	Arg	Val	Met	Ile	Leu	Lys	Total key AA
None	Ctrl	93.95	101.39	124.36	29.71	94.58	294.49	62.59	801.07	9.63	10.39	12.75	3.05	9.70	30.19	6.41	82.12
	AO	65.02	122.01	71.18	9.50	52.27	112.86	50.82	483.66	7.93	14.88	8.68	1.16	6.37	13.76	6.20	58.98
	RO	74.15	140.15	91.39	23.93	72.43	172.29	58.16	632.50	9.00	17.02	11.10	2.91	8.79	20.92	7.06	76.80
	MI	93.09	130.95	111.02	29.77	87.20	218.91	74.69	745.64	10.78	15.16	12.86	3.45	10.10	25.35	8.65	86.35
	TR	87.93	96.29	68.89	16.41	44.87	93.61	55.48	463.49	11.99	13.13	9.39	2.24	6.12	12.77	7.57	63.21
N-1%	Ctrl	91.65	109.09	124.52	31.17	95.42	287.75	50.15	789.74	9.29	11.06	12.62	3.16	9.67	29.17	5.08	80.05
	AO	70.31	115.77	82.07	11.08	57.86	129.99	47.56	514.65	8.51	14.00	9.93	1.34	7.00	15.72	5.75	62.25
	RO	81.12	136.17	104.93	28.84	81.68	216.80	53.80	703.33	9.42	15.79	12.19	3.35	9.49	25.19	6.24	81.67
	MI	105.66	143.35	135.43	39.70	107.87	295.89	76.67	904.57	11.83	16.05	15.16	4.45	12.08	33.13	8.58	101.27
	TR	104.42	108.88	94.18	24.98	63.26	141.33	57.31	594.36	13.98	14.58	12.61	3.34	8.47	18.93	7.68	79.60
N-2.5%	Ctrl	90.68	113.66	120.14	30.66	93.93	283.69	41.21	773.97	8.92	11.19	11.82	3.02	9.25	27.92	4.06	76.18
	AO	82.33	101.47	106.61	27.27	83.68	250.17	30.74	682.28	8.44	10.41	10.93	2.80	8.58	25.65	3.15	69.97
	RO	92.40	127.64	117.44	35.07	93.92	259.41	60.40	786.28	10.55	14.58	13.41	4.00	10.73	29.63	6.90	89.80
	MI	99.28	131.70	124.28	33.83	100.17	271.59	59.56	820.40	11.11	14.74	13.92	3.79	11.22	30.39	6.68	91.86
	TR	105.45	87.25	132.82	32.74	105.87	288.93	50.79	803.84	11.12	9.20	14.01	3.45	11.17	30.47	5.36	84.79
N-5%	Ctrl	99.58	117.24	128.84	30.52	98.85	290.38	33.88	799.28	9.30	10.94	12.03	2.85	9.23	27.11	3.16	74.61
	AO	107.92	121.66	133.51	31.37	105.31	302.14	34.56	836.47	10.29	11.60	12.73	2.99	10.04	28.80	3.29	79.74
	RO	100.32	141.05	124.00	31.36	100.20	271.21	40.82	808.95	10.63	14.95	13.14	3.32	10.62	28.75	4.33	85.74
	MI	118.22	156.78	142.47	38.02	112.91	317.04	50.51	935.96	12.06	15.99	14.53	3.88	11.52	32.34	5.15	95.47
	TR	96.93	113.82	125.03	31.11	100.84	279.96	38.64	786.32	9.29	10.91	11.98	2.99	9.67	26.79	3.72	75.35

^a AO: *A. oryzae* RO: *R. oryzae*, MI: *M. indicus*, TR: *T. reesei*.^b Thr: threonine, Arg: arginine, Val: valine, Met: methionine, Ile: isoleucine, Leu: leucine, Lys: lysine.

urea, only *M. indicus* showed remarkable improvement of total essential AA from 789.7 to 904.6 mg per initial dry substrate, an increase by 14.5%. Among them, Thr, Arg, Val, Met, Ile, Leu and Lys were improved by 15, 31, 9, 21, 13, 2.8, and 53%, respectively. In addition, Arg was increased in *A. oryzae* and *R. oryzae*, Thr was increased in *T. reesei*, Lys was increased in *R. oryzae* and *T. reesei*. As urea supply elevated to 2.5% N, *R. oryzae*, *M. indicus*, and *T. reesei* all showed improved total essential AA compared to Ctrl. With urea supply increased to 5% N, *A. oryzae*, *R. oryzae*, and *M. indicus* showed improved total essential AA. The highest total essential AA (936 mg per initial dry substrate) was observed in *M. indicus* with a 17.1% net essential AA improvement, contributed by enrichment of each essential AA. It was noticed that the yield of Lys and Arg changed in Ctrl treatment when urea concentration was increased (Table 2), which might be related to urea-amino acids interaction that caused protein denaturation (Stumpe and Grubmüller, 2007). The conversion of urea into amino acids could be due to the de novo synthesis of amino acids, where the fungal strains firstly catalyze urea into ammonia which is then utilized to form amino acids (Atasoglu and Wallace, 2002). It was reported that under Basal medium with trypticase, 59% and 73% of fungal amino acid N in *Neocallimastix frontalis* and *Piromyces communis*, and 68% and 87% of bacterial amino acid N in *Prevotella bryantii* and *Selenomonas ruminantium*, were derived from exogenous ammonia N (Atasoglu et al., 1998). In solid-state fermentation of present study, the fungal amino acids N could come from both de novo synthesis from urea N and hydrolysis of substrate protein.

It was noted in Table 2 that, regardless of urea supplement, *M. indicus* showed improvement for Thr, Arg, Met and Lys after fermentation compared to Ctrl. With 1% N urea addition, *M. indicus* improved Thr by 15%, Arg by 31%, Met by 27%, and Lys by 53%, higher than or similar to the treatments with 2.5% and 5.0% urea supplemented. Arg, Met, Lys, and Thr were commonly considered as limiting amino acids in animal diets (Lemme, 2009; Liao et al., 2015; Morris, 2007). Arg is regarded as conditionally essential AA which is typically sufficient in healthy animals but needs to be supplied with diet when catabolic stresses such as inflammation, infection, or dysfunction of kidney and small intestine occur (Morris, 2007). Significant ($p < 0.05$) improvement of Arg concentration after fermentation was observed in *A. oryzae*, *R. oryzae*, and *M. indicus* without urea supply, in all fungal strains when 1.0% N of urea was supplied, and in only *R. oryzae*, and *M. indicus* when urea supply increased from 2.5% N to 5.0% N. Met was considered as the first limiting AA for laying hens. It was reported that inclusion of Met from 2 to 6 g/kg feed could improve egg mass by two times (Lemme, 2009). The non-fermented substrate had around 3 g/kg d.b. of Met, which was significantly ($p < 0.05$) improved to 4.4 g/kg d.b. after fermentation with *M. indicus* when 1.0% N of urea was supplied. *R. oryzae* also improved ($p < 0.05$) Met from 3.0 to 4.0 g/kg d.b. when 2.5% N of urea was supplied. Thr is a limiting AA for growth of young broiler chicken. The maximum weight gain was achieved for broiler chicks fed with diet containing 25% crude protein with supplementation of Thr at 7.7 g/kg feed (Rangel-Lugo et al., 1994). Thr concentration in non-fermented substrate was around 9 g/kg d.b., which was significantly ($p < 0.05$) increased to 10.8 and 12.0 mg/g d.b., respectively, after fermentation by *M. indicus* and *T. reesei* without urea supply, and to 11.8 and 14.0 mg/g d.b., respectively, when 1% N of urea was supplied. Lys was considered as the first limiting AA in swine diets where it was used as substrate for gaining body protein, pep-

tides, etc., while excessive Lys were used as energy source (Liao et al., 2015). Lys concentration was significantly improved ($p < 0.05$) in fermented substrates by *M. indicus* without urea supply, by *R. oryzae*, *T. reesei* and *M. indicus* when 1.0% N and 2.5% N of urea were supplied, by *R. oryzae* and *M. indicus* when 5.0% N of urea was supplied. The highest concentration of total key AA was observed in *M. indicus* fermented substrate with 1.0% N of urea supplied (Table 2). Therefore, *M. indicus* could be considered as efficient fungal strain to improve key amino acids for swine and poultry nutrition.

3.2. Effects of different fungal strains on degradation of structural polysaccharides

The yield of structural polysaccharides (SP) in the substrates fermented by *A. oryzae*, *R. oryzae*, *M. indicus*, and *T. reesei* without and with different level of urea addition were shown in Fig. 2. The highest degradation of total SP was observed in *T. reesei* fermented substrate without and with addition of 1% N urea. Compared to non-fermented substrate (Ctrl), *T. reesei* without urea and with 1% N urea degraded total SP by 39.7% and 40.9%, respectively. However, when the urea addition was further increased to 2.5 and 5.0% N, no total SP degradation was noticed, indicating that urea at these levels had a severe inhibition on fiber utilization by *T. reesei*. A similar trend was clearly seen in profiles of glucan, xylan and arabinan. *T. reesei* was reported as a efficient producer of carbohydrase with cellulase and xylanase production induced when high-fiber substrate was present (Li et al., 2017) which is in agreement with the present study where mixing of high fiber feedstock SH with WDGS was used. Compared to *T. reesei*, other fungal strains showed negligible total SP reduction. Without urea addition, *A. oryzae*, *R. oryzae* and *M. indicus* degraded total SP by 10.2%, 15.2% and 3.9%, respectively. This degradation ability was reduced in *A. oryzae* and *R. oryzae* while remained unchanged in *M. indicus* when urea was added and increased. Ability for naturally production of cellulase and xylanase were reported in *A. oryzae* (Hoa and Hung, 2013), *R. oryzae* (Londoño-Hernández et al., 2017) and *M. indicus* (Karimi and Zamani, 2013), however, their fiber degradation ability was lower than *T. reesei*. It was noticed that without urea supplement (None), *T. reesei* consumed significant amount of SP (by 39.7%) in the substrate, but also consumed 29.8% of the total AA in the substrate as nitrogen source, resulting in overall decreased AA concentration (Fig. 1A). However, with 1% N of urea addition, *T. reesei* utilized similar amount of SP (by 40.9%), but consumed urea (Fig. 1B) as priority nitrogen source over substrate protein to synthesize amino acids for fungal biomass. Therefore, urea served as a substitution for substrate AA as nitrogen source which not only preserved substrate AA but also accumulated additional AA in fungal biomass. However, higher urea supplement (2.5% N and 5% N) in substrate severely inhibited *T. reesei*'s growth, resulting in blockade of cellulose and hemicellulose metabolisms. Similar inhibitory effect of higher urea supply was also found in fungus *Gongronella butleri* during chitosan accumulation (Nwe and Stevens, 2004). Therefore, 1% N of urea supplement would be appropriate if *T. reesei* will be used for fiber degradation and AA enrichment.

The soluble sugars containing total monosaccharides (glucose, xylose, galactose, arabinose and mannose) and cellobiose in each treatment after fermentation were shown in Fig. 3. The substrate mixture WDGS/SH (75/25) had around 11 mg/g of total monosaccharides and 7.5 mg/g of cellobiose. These soluble sugars were reduced gradually when increased

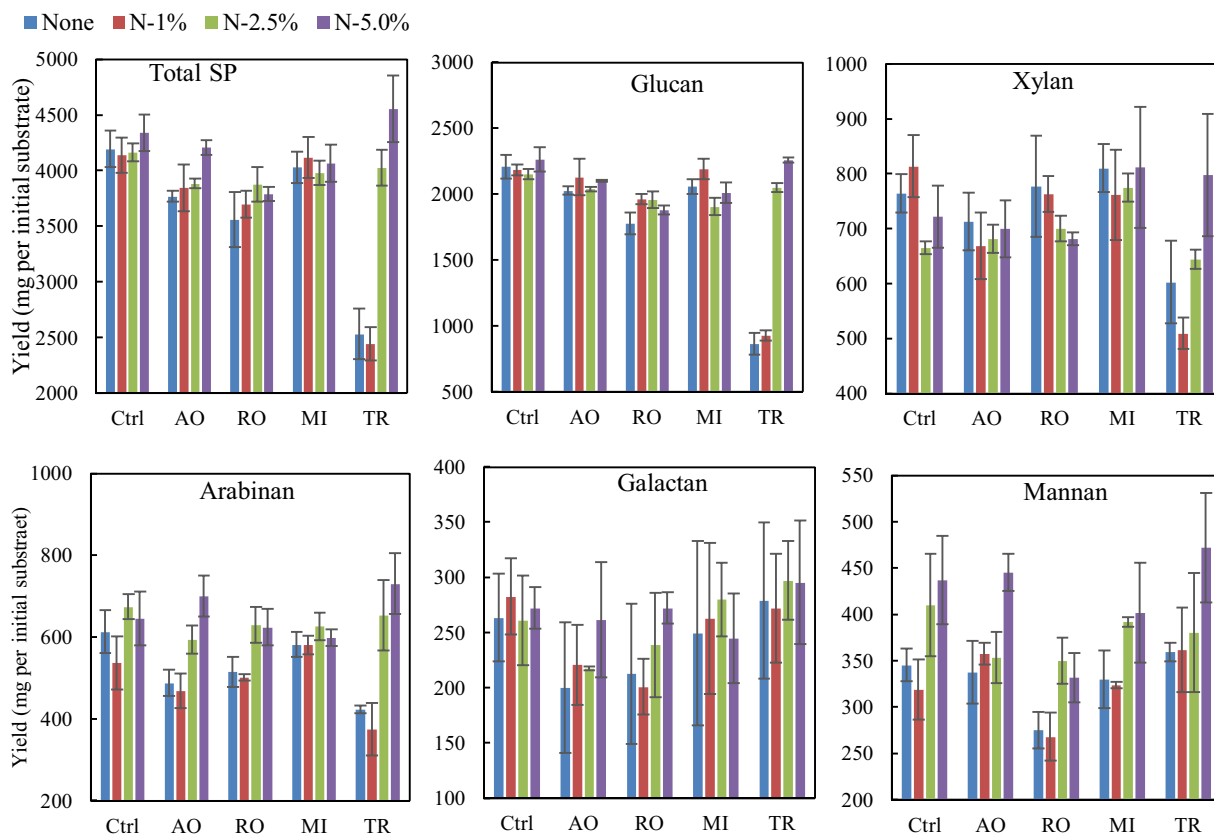


Fig. 2 – Yield of total structural polysaccharides (SP), glucan, xylan, galactan, arabinan and mannan in non-fermented (Ctrl) and *A. oryzae* (AO), *R. oryzae* (RO), *M. indicus* (MI), and *T. reesei* (TR) fermented WDGs/SH (75/25) mixture without (None) and with pre-addition of urea at 1.0, 2.5, and 5.0% N. All values are means with error bars representing standard deviations of three replications.

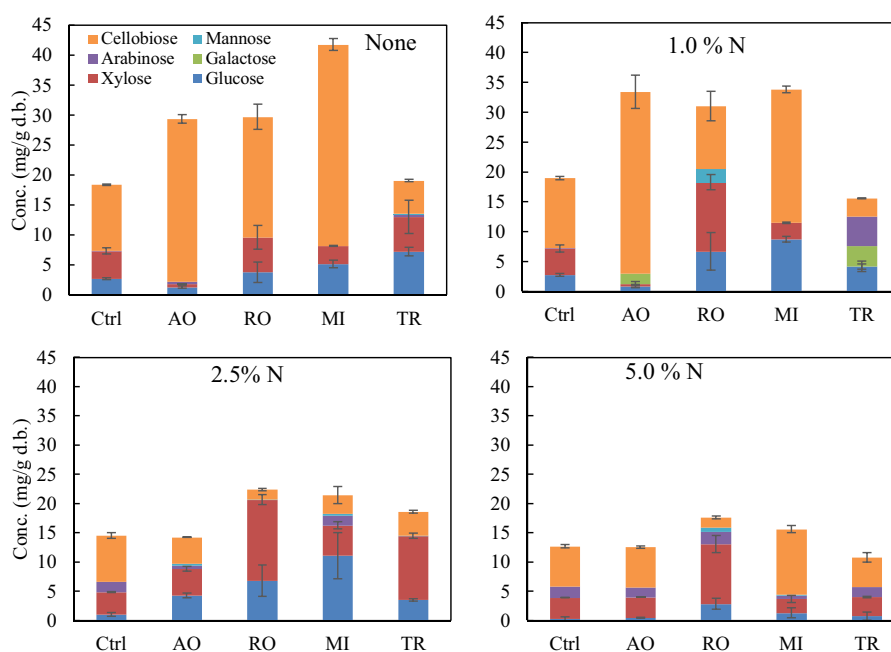


Fig. 3 – Different monosaccharides (glucose, xylose, galactose, arabinose, and mannose) and cellobiose concentration in non-fermented (Ctrl) and *A. oryzae* (AO), *R. oryzae* (RO), *M. indicus* (MI), and *T. reesei* (TR) fermented WDGs/SH (75/25) mixture without (None) and with pre-addition of urea at 1% N. All values are means with error bars representing standard deviations of three replications.

amount of urea was added, which could be due to possible interactions between the sugars and urea that form water-insoluble compounds (Imperato et al., 2005). The soluble sugars present in the substrate could be from unused

fermentable sugars remained in WDGs and new sugars generated during high-temperature sterilization process. Without urea supply (None), fermentation with fungal strains *A. oryzae*, *R. oryzae*, *M. indicus* showed increased production of

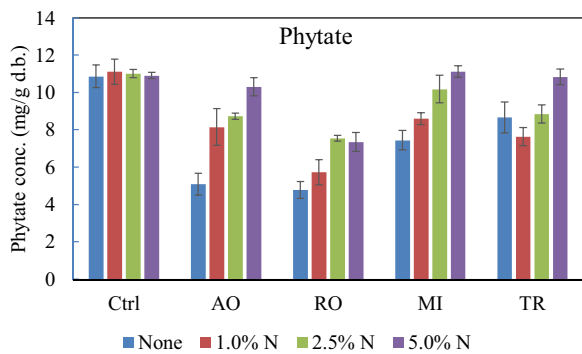


Fig. 4 – Phytate concentration in non-fermented (Ctrl) and *A. oryzae* (AO), *R. oryzae* (RO), *M. indicus* (MI), and *T. reesei* (TR) fermented WDGS/SH (75/25) mixture without (None) and with pre-addition of urea at 1.0, 2.5, and 5.0% N. All values are means with error bars representing standard deviations of three replications.

cellobiose, which could be due to catalysis of cellulose by exo-cellobiohydrolase and endo-glucanase synthesized by these fungal strains (Coughlan, 1991). Except for *A. oryzae* which utilized most of glucose and xylose present in the substrate, all other fungi accumulated certain amount of glucose which could be from hydrolysis of cellobiose by β -glucosidase produced by the fungi (Coughlan, 1991). *T. reesei* showed more production of glucose and xylose due to its strong ability of synthesizing β -glucosidase and xylanase (Li et al., 2017). With 1.0% N urea supply, higher production of glucose was found in *R. oryzae* and *M. indicus* and more xylose was accumulated with *R. oryzae* compared with non-urea treatments. *T. reesei* showed more galactose and arabinose production as compared to other treatments. It was noted that about 40% structural polysaccharides in substrate were decomposed by *T. reesei* (Fig. 2) but not many monosaccharides were accumulated compared to non-fermented substrate (Fig. 3), indicating the conversion of structural polysaccharides by *T. reesei* into carbon dioxide. With 2.5% N urea supply, there were still glucose and xylose produced by all fungal strains as compared to substrate without fermentation. However, cellobiose was no longer accumulated by the fungi which could be due to lack of exo-cellobiohydrolase and endo-glucanase synthesis or activities caused by growth inhibition. At 5.0% N urea, only *R. oryzae* and *M. indicus* showed some activity which can be seen from accumulated xylose and glucose with *R. oryzae* and increased cellobiose with *M. indicus*. Since glucose and xylose are two major fermentable sugars that can be directly assimilated in gastrointestinal tracts of monogastric animals, fermentation with *R. oryzae* and *M. indicus* with supply of 1.0 or 2.5% N urea would show advantages over non-fermented substrate in providing the easily digested sugars.

3.3. Degradation of anti-nutritional factors by different fungal strains

Phytate as one of the major anti-nutritional factors for monogastric animals is usually breakdown to improve utilization of phosphorous and feed digestibility by extraneous addition of phytase. This phytase can be produced by *A. oryzae* (Sapna and Singh, 2014), *R. oryzae* (Rani and Ghosh, 2011), *M. indicus* (Gulati et al., 2007) and *T. reesei* (Näsi et al., 1999). The degradation of phytate by different fungal strains in the substrate supplemented by different concentration of urea was

shown in Fig. 4. The substrate WDGS/SH (75/25) mixture contains around 11 mg/g of phytate, and no significant difference ($p > 0.05$) was observed in the substrate without and with urea supply. The degradation of phytate after fermentation reduced with increased urea supply in all fungal treatments. The highest degradation of phytate was observed in *A. oryzae* (by 53%), *R. oryzae* (by 56%), and *M. indicus* (by 31%) without urea supply, while in *T. reesei* (by 31%) with supply of 1% N urea. Supplementation of 5% N urea to the substrate resulted in no significant degradation of phytate by *A. oryzae*, *M. indicus*, and *T. reesei* except for *R. oryzae* which still showed 33% reduction of phytate. The phytate degradation without and with 1% N urea supply did not show significant difference ($p > 0.05$) in *R. oryzae*, *M. indicus* and *T. reesei*, while the phytate degradation by *A. oryzae* was more sensitive to urea supply and reduced significantly ($p < 0.05$) when 1% N urea was added. 2.5% or 5.0% N urea supply would be inhibitory to phytate degradation for all fungal strains used in this study. This could be on one hand due to less requirement of phytate degrading enzymes (phytase and phosphatase) which break phytate and release more nitrogen compounds in substrates for fungal growth and metabolisms when the readily available nitrogen source urea was present. On the other hand, urea as highly water-soluble compound could reduce water activity at high concentration, thus resulting in low free water for fungal growth and metabolism. Therefore, supply of 1% N urea would be appropriate for *R. oryzae*, *M. indicus* and *T. reesei* without compromise of phytate degradation.

The concentrations of three most common mycotoxins (total aflatoxin, zearalenone, deoxynivalenol) were monitored in the non-fermented and fermented substrates by four different fungi without and with supply of 1% N urea (Fig. 5). The total aflatoxin in the substrate mixture without fermentation were between 0.5 and 0.6 ppb, lower than the maximum allowable limit (20 ppb) for animal feed as established by U.S. Food Drug Administration (FDA, 2000). Therefore, the substrate as a feeding ingredient would not present a threat of aflatoxin to animals. It was noted that after fermentation, *R. oryzae*, *M. indicus*, and *T. reesei* in none-urea supplied treatments showed lower concentration of total aflatoxin than Ctrl treatment and treatments by the same fungal strains when 1% N urea was supplied. This indicates the potential for these fungal strains in detoxifying the total aflatoxins and that presence of urea may prohibit the detoxification ability of the fungi. However, the difference was not statistically significant ($p > 0.05$) which could be due to low initial concentration of the total aflatoxin in the substrate mixture. Degradation of aflatoxins was reported to be conducted by enzymatic reactions of extracellular fungal proteins which breakdown the cyclopentane ring of the aflatoxins, changing them into non-toxic compounds (Ji et al., 2016). Unlike other fungi, *A. oryzae* in this study produced around 0.3 ppb of total aflatoxin in both non-urea and urea added substrates, although the amount was not significant ($p > 0.05$). This could be due to genome similarity of *A. oryzae* and *A. flavus* which is well-known as aflatoxin producer (Payne et al., 2006).

Zearalenone (ZEN) concentration in non-fermented substrate was between 200 and 250 ppb (Fig. 5) which was above the maximum limit (200 ppb) for feeding young sows (Hurburgh et al., 2019). The three fungal strains *A. oryzae*, *R. oryzae* and *M. indicus* after fermenting the substrate showed significant ($p < 0.05$) reduction of ZEN by 59%, 25%, and 89%, respectively, without urea supply. Similarly, around 47%, 37%,

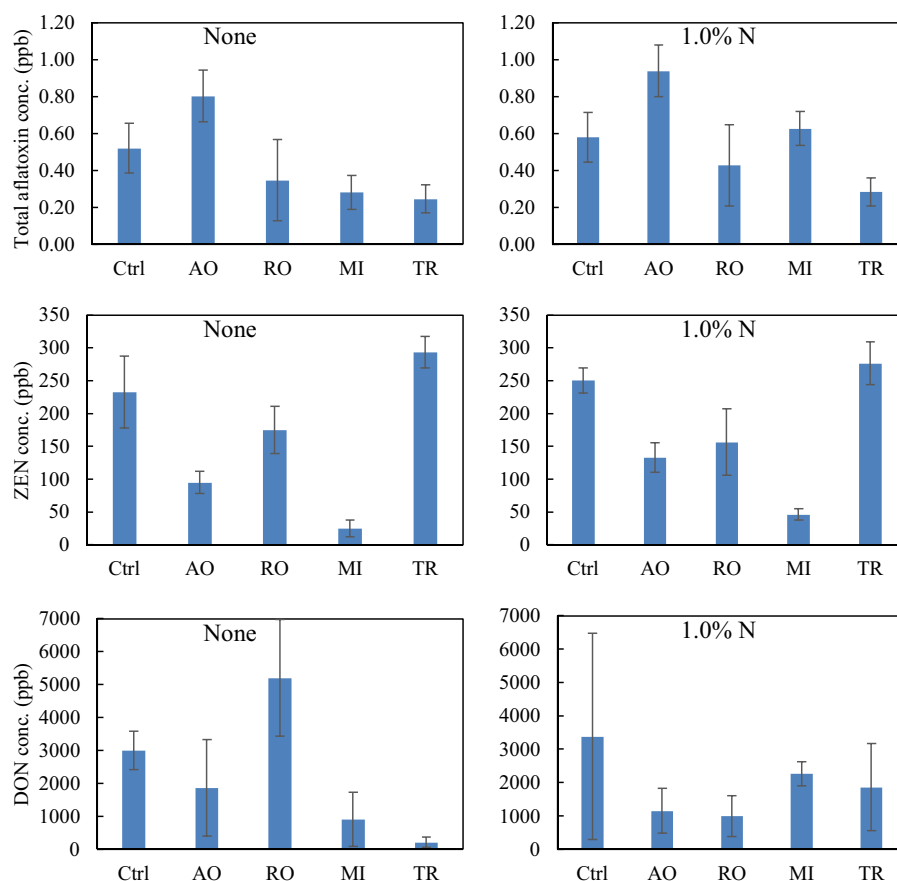


Fig. 5 – Concentration of total aflatoxin, ZEN, and DON in non-fermented (Ctrl) and *A. oryzae* (AO), *R. oryzae* (RO), *M. indicus* (MI), and *T. reesei* (TR) fermented WDGs/SH (75/25) mixture without (None) and with pre-addition of urea at 1.0% N. All values are means with error bars representing standard deviations of three replications.

and 81% of ZEN were significantly reduced ($p < 0.05$) by *A. oryzae*, *R. oryzae* and *M. indicus* with 1% N urea supply. The three fungal strains reduced ZEN concentration to levels below 200 ppb, making the fermented feed suitable for feeding young saws. However, *T. reesei* did not show significant reduction ($p > 0.05$) of ZEN for substrates regardless of urea supplement. ZEN can be effectively degraded and transformed by fungal strains under different metabolic pathways. For example, the lactonase produced by *Gliocladium roseum* can degrade lactone ring in ZEN which was further degraded by decarboxylation. *Thricosporon mycotoxinivorans* degraded ZEN by cleaving a macrocyclic ring in it. Certain *Rhizopus* sp. such as *R. arrhizus* had ability to catalyze sulfation of ZEN at C-4 hydroxyl group, forming a less toxic metabolite (Ji et al., 2016). As GRAS strains, *A. oryzae*, *R. oryzae* and *M. indicus* used in this study would have great potential in detoxification of ZEN in contaminated animal feeding ingredients.

Deoxynivalenol (DON) or vomitoxin in non-fermented substrate was around 3000 ppb (Fig. 5), higher than the 1000 ppb maximum limit for swine feed (Hurburgh et al., 2019). *A. oryzae*, *M. indicus*, and *T. reesei* had potential of detoxifying DON in substrate without urea supply, as compared to the Ctrl treatment. The detoxification ability of DON was declined with *M. indicus* and *T. reesei* but enhanced with *R. oryzae* when 1% N urea was supplied. *A. oryzae*, *M. indicus*, *T. reesei* had potential to reduce DON to below 1000 ppb in substrate without urea supply, while such goal can be achieved by *A. oryzae*, *R. oryzae* and *T. reesei* with 1% N urea supplement. The detoxification of DON by *R. oryzae* could be induced by urea. Compared to bacteria, not many fungal strains had been reported to detox-

ify DON. One fungal strain reported was *Aspergillus tubingensis* that can transform DON into metabolite that had molecular weight larger than that of DON (He et al., 2008).

The detoxification ability for total aflatoxin, ZEN and DON by each fungal strain was shown in Table 3. *T. reesei* had the highest ability to detoxify total aflatoxin in WDGs/SH (75/25) mixture without (by 53%) and with 1% N urea supply (by 51%). Moreover, *T. reesei* showed the highest detoxification of DON (by 93%) in the mixture without urea. *M. indicus* had relatively consistent degradation of ZEN by over 80% in the substrate both without and with urea supply. *R. oryzae* had the highest DON detoxification only in the substrate with 1% N urea supply. *A. oryzae* could be an efficient fungus for ZEN and DON detoxification, although it may still pertain some genome as *A. flavus* for producing aflatoxin. It is worth noting that *M. indicus* and *R. oryzae* were efficient to degrade all the three mycotoxins in substrate without urea and with 1% N urea, respectively, demonstrating the efficiency of these two fungal strains both technically and economically for animal feed detoxification. In addition, processing contaminated animal feed by a combination of two or more of these fungal strains both in a single process or sequential process could have potential of even higher degradation of all the mycotoxins, which is worth of further research.

Urea with its value usually underestimated, is commonly used as nitrogen fertilizer for crops. However, inappropriate irrigation with urea application could lead to its runoff and eutrophication in water bodies (Krausfeldt et al., 2019). As a low-cost nitrogen source, urea has advantages of being a nutrient for bacteria and fungi growth during biological pro-

Table 3 – Mycotoxin conversion of each fungal strains in substrate without and with 1% N supply of urea.

Mycotoxin ^a	None				1% N Urea			
	AO ^b %	RO	MI	TR	AO	RO	MI	TR
Total aflatoxin	–54.34 ^c	33.21	45.90	52.82	–61.95	26.25	–8.13	51.03
ZEN	59.16	24.85	89.19	–26.24	46.81	37.49	81.56	–10.42
DON	38.03	–73.29	69.64	92.87	65.97	70.55	33.02	44.90

^a ZEN: zearalenone, DON: deoxynivalenol.

^b AO: *A. oryzae*, RO: *R. oryzae*, MI: *M. indicus*, TR: *T. reesei*.

^c Negative mark represents either non-conversion or production of mycotoxin.

cessing (Navarathna et al., 2010). Combining urea in solid-state fermentation to process WDGS and SH mixture was demonstrated in present study to accumulate fungal amino acids and improve the overall amino acids profile of the fermented product with *R. oryzae* and *M. indicus*. Using *T. reesei* in the fermentation also reduced structural polysaccharides in the mixture, and resulting in low fiber feeding ingredient that is preferred by monogastric animals. Future study should focus more on combination strategies of culturing these protein enrichment fungi, fiber-reducing fungi, together with or without supply of phytase, for producing feed with higher protein and fiber digestibility. Existence of mycotoxins in agricultural produces is a global safety concern for both human food and animal feed. The capability of GRAS fungal strains to degrade mycotoxins may have remarkable significance in animal feed processing. In present study, *M. indicus* and *R. oryzae* showed potential of degrading total aflatoxin, ZEN and DON, indicating their economic value in detoxification of agricultural produces and therefore reducing economic loss due to discarding of contaminated feed and animal intoxication. More GRAS strains with detoxification ability that could be used for feed upcycling should be discovered and tested in future study. Given the high price of threonine (\$2.03 per kg), lysine (\$1.84 per kg), and tryptophan (\$7.60 per kg) (Feed, 2021), and low price of urea (\$0.245 per kg) (IndexMundi, 2021), the big profit margin by microbial conversion of urea to amino acids indicated a great economical potential of using urea as raw material for high amino acids feed production. As suggested in this study, degradation of phytate and mycotoxins may not be benefited with high concentration of urea supply. Therefore, careful consideration should be taken in future study based on types of fungal strains, substrates, incubation condition when using urea as nitrogen source.

4. Conclusion

Bioprocessing of WDGS and soybean hull mixture with fungal strains have potential of upgrading them to serve as better nutrition for swine and poultry. This study investigated the effect of urea supply on accumulation of total amino acids, essential amino acids, fermentable sugar, reduction of non-starch polysaccharides, and degradation of phytate and mycotoxins by four different fungal strains. It was found that 1% N urea supply could improve total AA yield by 13.3% with over 95% consumption of the supplied urea by *M. indicus*. At 1% N urea, around 41% of structural polysaccharides in substrate were degraded which concentrated AA by 19% with *T. reesei*. All four fungal strains *A. oryzae*, *R. oryzae*, *M. indicus* and *T. reesei* showed different degradation ability of phytate, total aflatoxin, ZEN and DON, demonstrating their remarkable potential for animal feed detoxification. This study demon-

strated feasibility of GRAS fungal strains in using urea to enrich amino acids while reducing fiber and degrading anti-nutritional factors, increasing feeding value of agro-industrial co-products for monogastric animals.

Conflict of interests

No conflict of interest is applied to this manuscript.

CRediT authorship contribution statement

Xiao Sun: Investigation, Writing - original draft, Writing - review & editing, Data curation, Methodology, Formal analysis. **Douglas G. Tiffany:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing. **Pedro E. Urriola:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing. **Gerald G. Shurson:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing. **Bo Hu:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

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.2021.09.004>.

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