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Feeding value improvement by co-fermentation of corn-ethanol co-product and agro-industrial residues with *Rhizopus oryzae*

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ABSTRACT

Corn dried distiller's grain with solubles (cDDGS) is a feeding ingredient for monogastric animals but with limited inclusion rate due to its imbalanced amino acids, high fiber content, and anti-nutritional compounds. Moreover, production of cDDGS is costly and energy intensive. This study investigated fungal fermentation of corn stillage mixing with other dry agro-industrial residues which could by-pass the energy-intensive drying process while improving nutritional value of the mixed substrates. *Rhizopus oryzae* was used to ferment WDGS blended with canola meal (CM), cottonseed meal (CSM), sugar beet pulp (SP) and soybean hull (SH) at different ratio at 28 ℃ for 12 days. Results suggested that short incubation period (4 days) was favorable for improvement of protein and key amino acids profile. Substrate mixture with C/N ratio above 3 effectively reduced ammonia generation. Significant reduction ($p < 0.05$) of structural carbohydrates (mainly glucan with degradation by 20–30 %) occurred after fermenting WDGS mixing with CM and SP. Substrate with all mixing ratio of SP or SH, and lower ratio (less than 50 %) of CM or CSM showed phytate degradation by 30–75 %. This study proved the feasibility of solid-state fermentation in improving feeding value of WDGS and agro-residues for monogastric animals.

1. Introduction

Nearly 94 % of total feedstock used for ethanol production in U.S. comes from corn grain (*Zea mays* L.), and 91 % of the corn grain is converted via dry grinding process to ethanol and co-products (mainly corn dried distiller's grain with solubles, or cDDGS) [1]. The cDDGS are used as feeding ingredient in mainly ruminant and monogastric animal feed, which is economically beneficial and contribute up to 25 % of total revenue for some corn ethanol plants [2]. However, the downstream processing of whole stillage (84–94 % moisture content) after ethanol distillation into cDDGS is energy intensive especially in the processes of evaporation and drying. Multiple strategies have been employed for water reuse and energy save. Examples including anaerobic digestion of thin stillage, where the generated water was used in fermentation and the produced biogas was collected for energy requirement during distillation and drying [3]. 70 % of total mass in corn grain is starch and is extracted as the form of ethanol and CO_2, resulting in the co-products consisting of highly concentrated protein, non-starch carbohydrates (mainly cellulose and hemicellulose) and phytate, as compared to corn grain. However, compared to soybean meal, proteins in cDDGS contain lower concentration of arginine (Arg), lysine (Lys), methionine (Met), and threonine (Thr), which were reported to be limiting amino acids in diets of swine, poultry and fish [4–6]. Imbalance of these amino acids in cDDGS limited its inclusion ratio in monogastric animal diet. Moreover, fiber and phytate are hard-to-digest components to monogastric animals, resulting in increased manure production that causes environmental concerns [7]. 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 [8]. Therefore, increasing key amino acids, reducing fiber and phytate in cDDGS has potential to raise its feeding value and increase benefits of corn-ethanol refineries.

Solid-state fermentation as one of the cost-effective bioprocessing has improved nutritional value of by-products from soybean, canola,

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cottonseed [9] and DDGS [10]. Mixing wet corn DGS (WDGS) with other agro-industrial residues could provide substrates with appropriate C/N ratio and moisture content for solid-state fermentation. This could potentially reduce energy cost in the evaporation and drying process for production of animal feed ingredient. The agro-industrial residues commonly produced in U.S. include canola meal, cottonseed meal, sugar beet pulp, soybean hulls, etc. Canola meal (CM) is a by-product from canola oil industry with increased production in U.S. from 16,000 metric ton in 1987 to around 1.2 million metric ton in 2020 [11]. Cottonseed meal (CSM) is by-product obtained after harvesting cotton fiber and oil by cotton farmers, and its production in U.S. is around 0.64 million metric ton in 2020 [11]. CM and CSM have protein content ranging between 35 and 45 % and are commonly used as protein-rich sources for swine and poultry diets [12,13]. However, their inclusion ratio are limited due to existence of glucosinolate and sinapic acid in CM and gossypol in CSM, both of which have negative effects on animal growth or reproduction system [14]. Soybean hull (SH) and sugar beet pulp (SP) are by-products from refineries of soybean and sugar beet with production in U.S. around 112 and 4.3 million metric tons in 2020, respectively [15]. SP and SH are rich in lignocellulosic fiber but low in protein, and commonly used as fiber-rich feed supplement in diets of both ruminant and monogastric animals [16,17]. Mixing these agro-industrial residues with WDGS could provide nutrient-rich substrate for fungal growth which could degrade the anti-nutritional compound and fiber content while improving amino acids profile favorable for mono-gastric animal diets. Economically, valorizing these agro-industrial residues could directly reduce their disposal cost, bringing potential revenues to the rural economy.

Fungi used for feed production are generally regarded as safe (GRAS). *Rhizopus oryzae* (*R. oryzae*) is commonly used fungal strain for production of human food (e.g. Tempeh in Indonesia) [18] and food-grade enzymes including protease, amylase [19], phytase [20], cellulase and xylanase [21]. These enzymes could change the chemical profiles of the substrate. Using hydrolytic enzymes in corn-ethanol fermentation had been reported to improve protein and amino acids digestibility of the resulting DDGS [22]. Moreover, *R. oryzae* used to ferment *Sophora flavescens* showed lactic acid production and remaining residues rich in protein that has potential as animal feed ingredient [23]. Utilization of *R. oryzae* to upcycle co-product from corn-ethanol plant mixing with the agro-industrial residues via solid state fermentation has not been reported, which could have potential to reduce co-products processing cost while meeting global feed protein demand [24]. In this study, WDGS mixing with CM, CSM, SP and SH at different mixing ratio were used as substrates for solid-state fermentation with *R.* oryzae. The appropriate incubation time, mixing ratio and type of mixing feedstock were evaluated by monitoring concentration or yield of crude protein, amino acids, ammonia accumulation. The reductions of structural carbohydrates and phytate were also evaluated.

2. Materials and methods

2.1. Feedstocks

WDGS was used to mimic the whole stillage after the ethanol distillation, and it was supplied by a dry-grinding corn (*Zea mays* L.) ethanol plant (Absolute Energy, St. Ansgar, IA). The WDGS upon received was stored frozen at -20 ℃ until use. Canola meal (CM) and cottonseed meal (CSM) were purchased from Seven Springs Farm, Check, VA, USA. Soybean hull (SH) and sugar beet pulp (SP) were purchased from Republic Mills, Inc., Okolona, OH, USA. The feedstocks CM, CSM, SH and SP were stored at dry and cool area upon received.

2.2. Fungal strains preparation

Rhizopus oryzae (*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. The spores of the fungus *R. oryzae* were preserved at -80 ℃ in 60 % (by volume) sterile glycerol solution. To activate *R. oryzae*, one drop of stored solution of spores was inoculated to the center of potato dextrose agar (PDA) petri-plate at 28 ℃ for 5 days (spores reached to around 1.3×10^7 CFU). Five pieces (each with size around 0.5 × 0.5 cm) of the developed *R. oryzae* mycelia and spores (around 2×10^5 CFU) on PDA medium were cut and transferred to 100 mL of freshly prepared and sterilized (121℃ for 20 min) potato dextrose broth (PDB) medium in each of 250 mL Erlenmeyer flasks. The sub-cultures of *R. oryzae* were cultured for 48 h at 28 ℃ in orbital shaker at 150 rpm to achieve the logarithmic phase of the strain shown as pelletized mycelia each with size of 1–5 mm.

2.3. Solid-state fermentation of WDGS/agro-industrial residue mixture with R. oryzae

WDGS mixed with each of CM, CSM, SH, or SP at different ratio could have different effects on crude protein and amino acids profile after fungal fermentation. *R. oryzae* was selected for fermentation of the mixtures due to its potential of fiber degradation [21] and amino acids improvement [25]. The dry weight of substrate mixture was kept at 10 g for each flask. The final moisture content of 70 % (w/w) in the mixture was achieved by adding deionized (DI) water, the amount of which was based on the moisture content in WDGS, each agro-industrial residue, and mixing ratio on dry basis. 70 % (w/w) moisture content of substrate mixture was selected due to its better performance with *R. oryzae* [26]. WDGS only (positive control) and WDGS mixing with each agro-industrial by-product (on dry basis) at 25 %, 50 %, 75 % and 100 % (negative control) was prepared in 250 mL Erlenmeyer flask with addition of appropriate DI water. The prepared substrate was then autoclaved at 121 ℃ for 15 min. The chemical composition such as protein and amino acids in substrate didn't subject to noticeable changes before and after sterilization (121 for 15 min) as reported with soybean meal [27]. The sterilized substrate was cool to room temperature prior to inoculation. Sterilized pipettor (Eppendorf, Enfield, CT, USA) were used to inoculate substrate in each fermentation flask with 5 mL (inoculation rate 15 % v/w) of pelletized *R. oryzae* mycelia (estimated to have 1×10^4 CFU) from the freshly prepared sub-culture (48 h inoculum age). The inoculated substrate was hand-shaken gently to spread the fungal mycelia as much as possible. All the flasks were incubated statically under 28 ℃ for 12 days. Total twelve replications for each treatment were performed and three bottles (n = 3) were withdrawn each time at 4 d, 8 d and 12 d. The samples collected were homogenized via stir mixing and dried in hot air dryer at 60 ℃ for 48 h to avoid degradation of amino acids and protein. The dry weight of total sample in each flask was measured to determine weight loss during fermentation (the difference of dry weight between fermented substrate at 4, 8, or 12 d and non-fermented substrate at 0 d). The dried samples were grinded to fine particles and stored in -20 ℃ freezer for further analysis.

2.4. Analytical methods

2.4.1. Proximate and crude protein analysis

Moisture content, total solids and ash content were determined based on procedures from National Renewable Energy Laboratory (NREL) [28]. Moisture content was determined based on the weight difference of the sample before and after drying in oven at 105 ℃ for 24 h. Total solids were defined as remained solids after moisture removal. Ash content at dry basis was determined by the weight remained of the dry sample (after 105 ℃ drying) after burning in 550 ℃ Maffle furnace for at least 4 h. Particle size distribution (% by weight as received) of each feedstock used was obtained by sieving each sample through US Standard Sieves No. 10 (mesh size of 2 mm), 18 (1 mm), 35 (0.5 mm), 60 (0.25 mm), and 120 (0.125 mm). Total crude protein were determined using DK20 automatic Kjedahl Digestion Unit (VELP Scientifica, Inc.,

Bohemia, NY) followed by distillation in UDK129 Distillation Unit (VELP Scientifica, Inc., Bohemia, NY) based on Kjeldahl method [29]. The crude protein was calculated using organic nitrogen to protein conversion factor of 6.25. The organic nitrogen was obtained by subtracting total ammonia nitrogen (TAN) from total Kjeldahl nitrogen (TKN).

2.4.2. Analysis of structural carbohydrates, amino acids, reducing sugar and phytic acid

Structural carbohydrates (glucan, xylan, arabinan, galactan, mannan) of the samples before and after fermentation were determined with two-step acid hydrolysis method based on NREL protocol [30]. The hydrolyzed samples containing monomeric sugars (glucose, xylose, arabinose, galactose, and mannose) after filtration with 0.22 mm PTFE filter were determined with HPLC (1200 Infinity series, Agilent Technology, Santa Clara, CA, USA) equipped with Biorad Aminex HPX-87 P analytical column (300 × 7.8 mm) with operating temperature of 80 °C, refractive index detector (RID) with operating temperature of 55 °C, and ultra-pure water as mobile phase at flow rate of 0.6 mL/min. The polymeric sugars were converted from the monomeric sugars using an anhydro correction of 0.88 for C5 sugars (xylose, arabinose) and a correction of 0.9 for C6 sugars (glucose, galactose, mannose).

The dried and ground solid samples were hydrolyzed to breakdown protein into amino acids. The hydrolysis of each sample (50 mg) was performed using 1.0 mL of 6 M HCl in 2 mL sealed centrifuge tube at 110 ℃ for 24 h. The headspace of each tube was purged with pure nitrogen before hydrolysis to avoid oxidation of sulfur-containing amino acids. The hydrolyzed samples were diluted and filtered through 0.22 μm PTFE filter before quantification. The analysis was performed in 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.) with operating temperature of 40 °C, and Diode array detector (DAD) using UV light source with wavelength of 338 nm and reference wavelength of 390 nm. 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 [31]. Two mobile phases were used. Mobile phase A contains (/L): 10 mmol Na_2HPO_4, 10 mmol $Na_2B_4O_7$, 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. The total flow rate of mobile phase during operation was 1.5 mL/min and running time for each sample was 25 min. Amino acids standards were prepared and calibrated based on procedures described [31].

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 [32]. Reducing sugar in this study indicates all monosaccharides that are soluble in water such as glucose, xylose, galactose, arabinose, mannose, etc. Reducing sugar in non-fermented and fermented substrates was determined based on DNS (3,5-dinitrosalicylic acid) method [33].

2.5. Calculation and statistical analysis

The C/N ratio of each raw substrate mixture was calculated by dividing the total nitrogen (TKN) from the total carbon in the structural carbohydrates of each substrate mixture on a dry weight basis. The carbon content in each structural carbohydrate (glucan, xylan, galactan, arabinan and mannan) is estimated as 40 % (by weight).

The statistical analysis was performed with Tukey's multiple comparison of means at 95 % confidence interval (p-value < 0.05) using JMP Pro 14.0.0 (SAS Institute Inc., Cary, NC, USA). The statistical analysis was used to determine pairwise statistical differences ($p < 0.05$) of structural carbohydrates, crude protein, ammonia, and amino acids. Data in figures and tables were presented as mean ± standard deviation.

3. Results and discussion

3.1. Composition of feedstock before solid-state fermentation

The proximate analysis, chemical composition and particle size distribution of each feedstock used are shown in Table 1. CM and CSM were oil cakes after oil extraction from canola and cottonseed, respectively, and contained high concentration of protein (around 40 % dry basis). The crude protein content on dry basis in CM and CSM was 31.8 % and 41.8 %, respectively, higher than that in WDGS. Total amino acids content in CM and CSM was 29.0 % and 5.3 %, respectively, higher than it in WDGS. The crude protein and amino acids profile of CM are consistent with the values published by Canola Council of Canada [34]. The protein and amino acids profile of CSM were also consistent with published results [35]. Particle with size between 0.25 and 2 mm accounted for over 70 % of the total weight of each feedstock. Except for CSM and SP, over 50 % of the weight for WDGS, CM and SH fall into the size between 0.5 and 1 mm (Table 1). It was noticed (Table S1, Supplementary material) that arginine (Arg), glutamate (Glu) and hydroxyproline (Hyp) accounted for 40.4 % and 45.4 % of total amino acids in CM and CSM, respectively. However, this proportion was only 31.5 % in WDGS. CM contains higher level of threonine (Thr), methionine (Met) and lysine (Lys) than CSM and WDGS, while CSM had higher content of Arg than CM and WDGS. The total amino acids concentration in fungus *R. oryzae* after 4-day growth in PDB medium was 224 mg/g dry biomass, lower than it in WDGS, CM and CSM (Table 1). *R. oryzae* cannot be considered as protein-rich biomass, but it contains relatively high concentration of Arg, Lys, and asparagine (Asp) which can be used as identifier for the fungal biomass in the fermented substrates (Table S1). SH and SP are rich in lignocellulosic components. SH consists of around 59.7 % of total structural carbohydrates which contain 38.5 % glucan and 21.1 % other polysaccharides (Table S2, Supplementary material), similar as reported (28.6 % cellulose and 20.0 % hemicellulose) [36]. SP consists of around 46.3 % of total structural carbohydrates that contain 26.3 % glucan and 20 % other polysaccharides (Table S2), similar as reported (20–25 % cellulose and 25–36 % hemicellulose) [37]. Both SH and SP had low protein content (up to around 10 % dry basis) and their total amino acids contents were only 26–32% that of WDGS. Most of the amino acids in SH and SP were below 5 mg/g dry substrate. Phytic acid contents in CM and CSM were two times higher than WDGS, while in negligible amount in SP and SH (Table 1).

Table 1
Proximate analysis, chemical composition and particle size distribution of each raw feedstock used in this study.

Parameter [a]	WDGS [b]	CM	CSM	SP	SH
Moisture content, % w.b.	49.1	8.8	15.9	7.2	9.2
Total solid, % w.b.	50.9	91.2	84.1	92.8	90.8
Ash, % d.b.	4.4	7.5	11.2	12.2	5.6
Crude protein, % d.b.	30.8	40.6	40.8	9.1	10.4
Total amino acids, mg/g d.b.	237.4	306.2	324.2	62.3	76.3
Total structural carbohydrates, % d.b.	36.7	24.7	21.1	46.3	59.7
Phytic acid, mg/g d.b.	14.6	28.0	28.4	0.27	0.42
Particle size distribution, % w.b.					
>2 mm	9.2	0.8	13.0	2.5	0.1
1–2 mm	34.6	10.5	31.1	21.6	25.1
0.5–1mm	51.6	51.4	26.7	33.9	58.5
0.25–0.5 mm	4.5	21.4	15.7	18.3	10.8
0.125–0.25 mm	0.2	13.4	9.8	12.6	4.4
< 0.125 mm	0	2.5	3.7	11.0	1.1

[a] d.b.: dry basis; w.b.: wet basis.
[b] WDGS, wet distillers grains with solubles; CM, canola meal; CSM, cottonseed meal; SP, sugar beet pulp; SH: soybean hull.

3.2. Effects of substrate mixture on crude protein, TAN, and amino acids profile during solid-state fermentation by R. oryzae

The changes of crude protein during fermentation of *R. oryzae* in different substrate mixtures are featured in Fig. 1. The higher crude protein in CM (40.6 %) and CSM (40.8 %) and lower crude protein in SP (9.1 %) and SH (10.4 %) than in WDGS resulted in the overall crude protein in their mixture with WDGS increased or decreased, respectively, with raised mixing ratio. Fermenting WDGS for 4 days significantly ($p < 0.05$) increased protein content by 25 %. Similar protein improvement by fermentation was also reported on fermented soybean meal (by 16.5 %) by *A oryzae* for 36 h [27] and fermented sweet potato and peanut residues (by 48.4 %) by co-culture of *A. oryzae* and *Bacillus subtilis* for 72 h [38]. Longer fermentation period was not benefitial for crude protein improvement. No change of crude protein content was observed from day 4 to day 8 in WDGS, followed by 20 % decreae from day 8 to day 12. The decrease of protein during late stage of fermentation could be due to protein metabolisms by the *R. oryzae* [39] which was involved when the hyphae of growing fungi penetrating the protein-rich substrate.

Significant improvement ($p < 0.05$) of crude protein was also observed in WDGS mixing with 50 % (by 13.2 %) of CM after 4 days of fermentation (Fig. 1). In 25 %, 75 % and 100 % CM substate, however, no significant ($p > 0.05$) protein accumulation was observed throughout 12 days of incubation. Mixing WDGS with increased ratio of CSM from 25 to 100 % did not show significant enrichment ($p > 0.05$) of crude protein after 4 days of fermentation. Fermentation of WDGS/SP mixture had enriched crude protein ($p < 0.05$) by 28.6 % only when mixing ratio of SP was 50 %. No improvement of crude protein was observed for treatments with mixing ratio of SH from 25 % to 100 %. However, the crude protein (N x 6.25) usually overestimate actual protein content due to existance of chitin (containing N) in fungal cell wall [40]. Since no extragenous nitrogen source was added, the improved crude protein concentration could be due to degradation of fiber and phytate that forms complex with protein.

No TAN was detected in all raw feedstocks used in this study (data not shown). Therefore, the TAN accumulated was exclusively due to fermentation process when protein and organic nitrogen compounds were metabolized. Less than 1.0 mg/g or 1000 ppm of TAN was accumulated after 4 days of fermentation in WDGS and WDGS mixing with SP and SH. This could be due to low protein content of SP and SH, and high C/N ratio in WDGS/SP and WDGS/SH mixture (Fig. 2). However, when the fungal hyphae reached proteinaceous part and occupied void spaces between particles of the substrate, ventilation reduced and anaerobic conditions in the substrates were created. These could cause more substrate protein metabolisms and lysis of fungal biomass protein, resulting in ammonia accumulation. In contrast, more than 2.0 mg/g of TAN was produced after 4 days in WDGS mixing with CM and CSM, which could be due to high protein content and low C/N ratio in the substrate mixture (Fig. 2) where protein metabolism occurred early during fungal growth. All the treatments showed increased TAN concentration after 8 and 12 days of fermentation. The TAN was formed from breakdown of proteins into peptides and amino acids followed by metabolisms of amino acids into ammonia which can be accelerated under anaerobic condition at later stage of fungal growth. Anaerobic condition could be alleviated by intermittent stirring of fermented substrate but may also cause disruption of mycelial attachment to solid substrate [41]. C/N ratio in the substrate played important role in metabolisms of fungal strain. It was indicated that higher C/N ratio of substrate caused less generation of TAN. Regardless of substrate mixture, the C/N ratio larger than 3.0 showed lower TAN than it with C/N ratio below 3.0, after 4 days of fermentation by *R. oryzae* (Fig. 2). The higher C/N ratio can be achieved by mixing WDGS with high fiber substrate such as SP and SH. Ammonia was commonly considered toxic to microorganisms if at higher level [42]. It was reported that TAN exceeding levels between 2000–7000 mg/L (ppm) had different inhibitory effects on microorganisms during anaerobic digestion [42]. In addition, excesive ammonia if fed to mono-gastric animal could lead to damage to the epithelial cells and therefore retarded growth [43]. However, mixing with higher fiber substrate means lower protein content in the final fermented product as shown in Fig. 1. Therefore, whether mixing protein rich or fiber rich residues with WDGS depends on the actual amount of protein and TAN generated after fermentation. In the present study, WDGS alone and its mixture with 50 % SP as substrate resulted in low TAN (less than 2000 ppm) and significantly improved ($p < 0.05$) crude protein content after 4 days of solid-state fermentation (Fig. 1).

The total amino acids concentration of each substrate mixture during

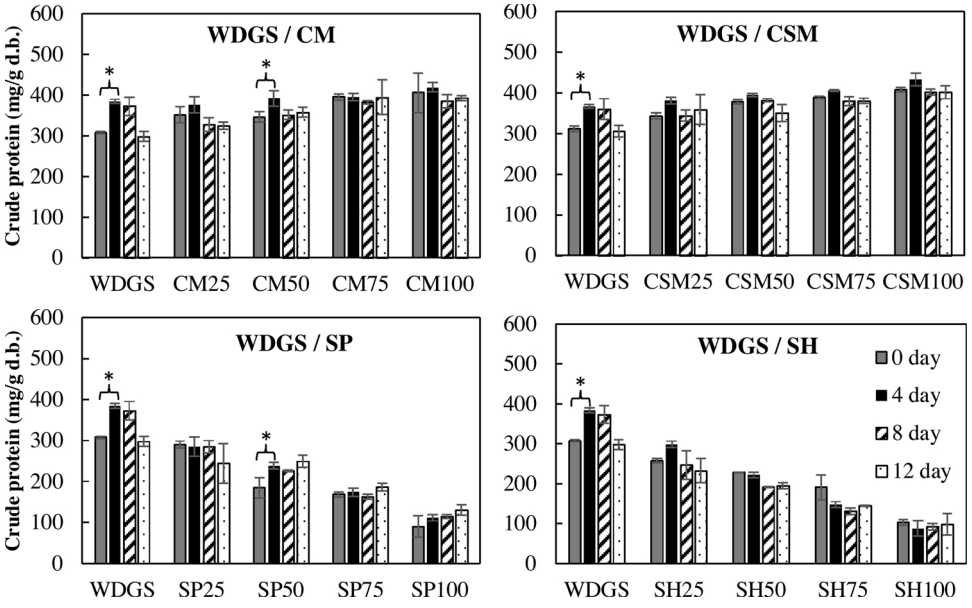

Fig. 1. Crude protein content of WDGS mixing with canola meal (CM), cotton seed meal (CSM), sugar beet pulp (SP), and soybean hull (SH) at 0, 25, 50, 75 and 100 % mixing ratio at 0 day without inoculation, after processing with *Rhizopus oryzae* for 4, 8, and 12 days. Star mark represents significant ($p < 0.05$) improvement between treatments.

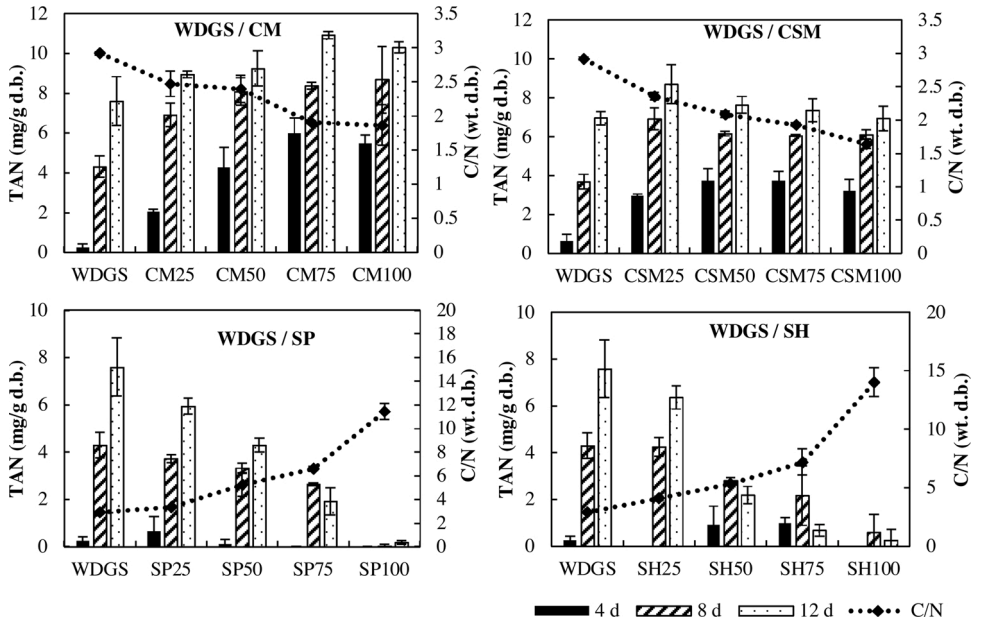

Fig. 2. Total ammonia nitrogen (TAN) content of WDGS mixing with canola meal (CM), cotton seed meal (CSM), sugar beet pulp (SP), and soybean hull (SH) at 0, 25, 50, 75 and 100 % mixing ratio after processing with *Rhizopus oryzae* for 4, 8, and 12 days. Solid diamond with dashed line represents the the C/N ratio at dry weight basis in the raw substrate mixture.

fermentation was shown in Fig. 3. The total amino acid content was lower than total crude protein (Table 1) which was due to existence of non-protein nitrogen in the feedstock and fungal biomass. Therefore, total amino acids would be more accurate to reflect the nutrient changes of substrate during fermentation. Based on the feedstock composition (Table 1), the total amino acids account for 77.13 % of crude protein in WDGS, 75.74 % in CM, 74.3 % in CSM, 68.76 % in SP, and 73.36 % in SH. The total amino acids increased by 3.7–15.3% in the substrate with increased mixing ratio of CM from 25 to 75 %. The total amino acids in *R. oryzae* were lower than WDGS, CM and CSM (Table S1), therefore, would not contribute to the increase of total amino acids in each substrate mixture after inoculation. The dilution effect of amino acids in *R. oryzae* should also be neglected due to relatively low inoculation rate (15 % v/w) of fungal biomass compared to the mass of substrate. However, the fermentation process didn't significantly improve the total amino acids concentration during the first 4 days. The total amino acids didn't significantly change ($p > 0.05$) in WDGS substrate from 0 day to 8 days, while significantly decreased by 27.8 % after 12 days fermentation compared to the initial condition (0 d). When increasing the mixing ratio of CM from 25 to 100 %, no significant increase ($p > 0.05$) was observed after 4 days of fermentation, while significant reduction ($p < 0.05$) by 25.6, 27.7, 35.4, and 23.5 % was found in substrate with CM mixing at 25, 50, 75 and 100 %, respectively, after 12 days of fermentation compared to the substrate before fermentation (0 d). It can be indicated that the decreased total amino acid was due to mineralization of amino acid into either ammonia or ammonium (TAN) by the fungal strain at

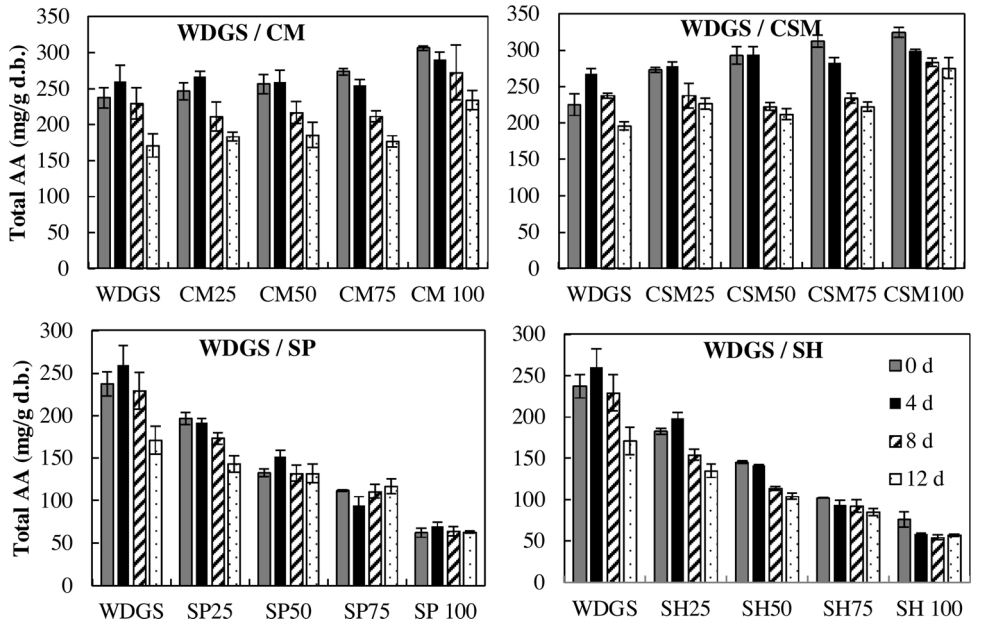

Fig. 3. Total amino acids (AA) of WDGS mixing with canola meal (CM), cotton seed meal (CSM), sugar beet pulp (SP), and soybean hull (SH) at 0, 25, 50, 75 and 100 % mixing ratio at 0 day without inoculation, after processing with *Rhizopus oryzae* for 4, 8, and 12 days.

mid and later stage of fermentation (from 4 d to 12 d) (Fig. 1). The same trend was applied to WDGS mixing with CSM. Similar as WDGS/CM, the total amino acid content reduced by 16.9, 27.4, 28.8 and 15.1 % after 12 days of fermentation compared to 0 d when mixing ratio of CSM increased to 25, 50, 75, and 100 %.

In substrate of WDGS mixing with increased ratio of SP and SH, the total amino acids content reduced due to lower protein content in SP and SH than in WDGS. Fermentation with the fungus *R. oryzae* did not improve total amino acids on mixture of WDGS/SP at various mixing ratio. Significant reduction ($p < 0.05$) of total amino acids was observed in mixture with 25 % of SP while no significant reduction was in substrate with 50–100% of SP. WDGS with 25 % SH has significant improvement ($p < 0.05$) of total amino acids during 4 days of fermentation, followed by significant reduction from day 4 to day 12. Fermenting the mixture for 4 days could improve total amino acids only in certain mixing residue and ratio (CM25, SP50 and SH 25), but the changes are not significant ($p > 0.05$). The results were consistent with another study where protein was significantly improved while total amino acids did not after fermenting soybean meal with *A. oryzae* [44]. It was believed that the increase in protein content was due to increase of selected amino acids rather than increase of all amino acids [44]. The increased amino acids concentration could be due to either convertion of non-protein nitrogen to fungal biomass or reduction of other components (fiber and phytate) in the substrate. It would be anticipited that total amino acids would be improved by supply of additional nitrogen sources, similar to a study where adding extraneous inorganic nitrogen source such as ammonium sulfate had shown remarkable improvement of crude protein (mg/g: unfermented 66.0, fermented without N source 110.8, fermented with N source 155.4) in fermented yam peal with *Saccharomyces cerevisiae* [45]. Supplementation of nitrogen source or pretreatment should be employed to provide more available nutrients for fungal fermentation if SP and SH would be processed further for protein-rich animal feed.

The balance of amino acids in monogastric animal diets is critical as recent studies showed that pigs strongly preferred diets with balanced amino acid than diets with excessive amino acid [46]. As shown in Table 2, WDGS alone after fermentation had improvement of Arg and Met proportion by more than 10 %. WDGS mixing with CM at 25, 50 and 75 % after fermentation showed improvement of Met proportion by over 10 %. Improvement of Arg proportion by over 10 % was also observed in WDGS/CSM, WDGS/SP, and WDGS/SH mixture at all mixing ratios. In addition, WDGS mixing with SP at 25 and 50 % showed enhanced Met and Lys proportion by over 10 %. The enrichment of these amino acids was from conversion of other amino acids (those with declined proportion after fermentation) or non-protein nitrogen by fungal catabolism and metabolism. Among these enrichments, Thr, Arg, Met, and Lys are the most prominent which indicates that they are the most common amino acids in the biomass of *R. oryzae* as shown in Table S1. Studies had shown that diets with excessive of Thr, Arg, or Lys are more preferred by pigs than the diets with excessive of Met or Trp [47]. Thr and Lys are the only essential amino acids that needs to be provided externally, because all other amino acids can either be synthesized from non-protein nitrogen or other amino acids [48]. Therefore, co-fermentation of substrate mixtures by *R. oryzae* could be an effective aproach to improve essential amino acids proportion in the feed.

3.3. Effects of fungal solid-state fermentation on degradation of structural carbohydrates

The total structural carbohydrates and reducing sugar of each substrate mixture before and after 4 days of fermentation were shown in Fig. 4. Higher fiber content in SH and SP and lower fiber content in CSM and CM compared to WDGS resulted in increased and decreased structural carbohydrates content in the substrates when the proportion of these feedstock increased (Fig. 4A). Fermentation of WDGS (W100) and WDGS mixed with SH (SH25, 50, 75, and 100) after 4 days by *R. oryzae* did not shown significant reduction ($p > 0.05$) of total structural carbohydrates, although a slight reduction was observed on WDGS only. In WDGS and SP mixture, the mixing ratio of SP at 25 % (SP25) and 75 % (SP75) showed significant reduction ($p < 0.05$) of total structural carbohydrates by 6.7 % and 10.2 %, respectively, after fermentation. When WDGS was mixed with CSM, significant reduction ($p < 0.05$) of total structural carbohydrates (by 12.3 %) was observed in the mixture with 75 % of CSM. When CM was mixing with WDGS, the total structural carbohydrates was significantly reduced ($p < 0.05$) by 6.6 %, 12.2 %, 19.9 % and 16.5 % in WDGS mixtures with 25 %, 50 %, 75 % and 100 % CM, respectively. The results indicated that fermenting WDGS and CM mixture by *R. oryzae* could have more effect on fiber degradation than fermenting other WDGS mixtures. In the non-fermented substrates, CM, CSM and SH had lower reducing sugar than WDGS. However, SP contained higher reducing sugars than WDGS (Fig. 4B), which could be due to degradation of remaining sucrose (non-reducing sugar) into glucose and fructose (reducing sugars) during sugar beet processing. The increased reducing sugars in substrates after 4 days fermentation was due to hydrolysis of structural carbohydrates in substrates (Fig. 4A) by *R. oryzae* that can produce a set of hydrolases such as cellulase,

Table 2
Key amino acids to total amino acid ratio in fermented and non-fermented substrate mixture after 4 days by *R. oryzae*.

Substrate	Ratio	Thr		Arg		Val		Met		Ile		Leu		Lys		Total key amino acid	
		N*	F*	N	F	N	F	N	F	N	F	N	F	N	F	N	F
WDGS	100	4.44	4.58	**4.96**	**6.02**	5.66	5.55	**1.56**	**1.89**	4.44	4.45	13.92	12.41	3.40	3.68	38.38	38.57
WDGS/CM	75/25	4.16	4.33	7.36	7.33	5.52	5.38	**1.61**	**1.95**	4.25	4.33	11.84	11.59	3.60	3.80	38.34	38.70
	50/50	3.95	4.18	9.33	8.57	5.31	5.35	**1.59**	**1.76**	4.03	4.30	9.89	10.10	3.95	4.05	38.07	38.32
	25/75	3.80	3.92	10.98	9.17	5.19	5.33	**1.35**	**1.67**	3.96	4.20	8.48	8.29	4.10	4.38	37.85	36.95
	0/100	3.70	3.82	12.68	11.80	5.00	5.15	1.31	1.43	3.76	3.95	7.08	7.01	4.26	4.23	37.78	37.39
WDGS/CSM	75/25	4.03	3.94	**6.22**	**8.11**	4.65	4.43	1.90	1.94	3.48	3.43	11.73	11.36	3.14	2.89	35.14	36.11
	50/50	4.02	4.01	**6.97**	**8.66**	4.39	4.33	2.05	1.90	3.35	3.39	10.10	9.67	3.34	3.15	34.23	35.12
	25/75	4.02	3.83	**7.43**	**8.16**	4.17	4.23	2.14	2.14	3.28	3.29	8.40	7.95	3.83	3.69	33.28	33.30
	0/100	4.36	4.43	**7.93**	**8.55**	4.30	4.36	1.98	1.68	3.39	3.42	7.39	7.22	5.10	4.42	34.44	34.07
WDGS/SP	75/25	3.98	4.40	**5.36**	**7.54**	4.79	4.88	**2.13**	**2.47**	3.47	3.54	12.64	10.37	**2.15**	**2.64**	34.52	35.85
	50/50	4.33	4.46	**5.57**	**7.17**	5.20	5.06	**2.22**	**2.56**	3.59	3.60	12.45	10.16	**2.13**	**2.43**	35.50	35.45
	25/75	4.83	4.78	**6.80**	**8.51**	5.45	5.35	2.03	1.86	3.78	3.52	9.59	9.12	**2.35**	**2.71**	34.83	35.86
	0/100	5.76	5.67	**5.59**	**6.29**	7.31	6.72	**1.28**	**1.68**	4.06	4.14	8.76	8.85	3.50	2.83	36.27	36.19
WDGS/SH	75/25	4.01	4.08	**5.33**	**8.16**	4.65	4.41	1.77	1.87	3.45	3.30	12.55	10.83	3.09	3.01	34.84	35.66
	50/50	3.98	3.95	**5.45**	**8.65**	4.68	4.46	1.75	1.83	3.38	3.27	11.93	9.33	3.25	3.45	34.42	34.94
	25/75	4.08	3.82	**5.06**	**8.80**	4.65	4.20	1.62	1.75	3.35	3.02	10.23	7.56	4.32	3.50	33.31	32.66
	0/100	3.84	4.02	**4.43**	**6.96**	4.30	4.88	**0.89**	**1.29**	3.17	3.40	**6.16**	**7.06**	5.94	4.64	28.74	32.25

Highlighted values represent at least 10 % improvement of key amino acid-to-total amino acid ratio after fermentation.
* N: non-fermented; F: fermented.

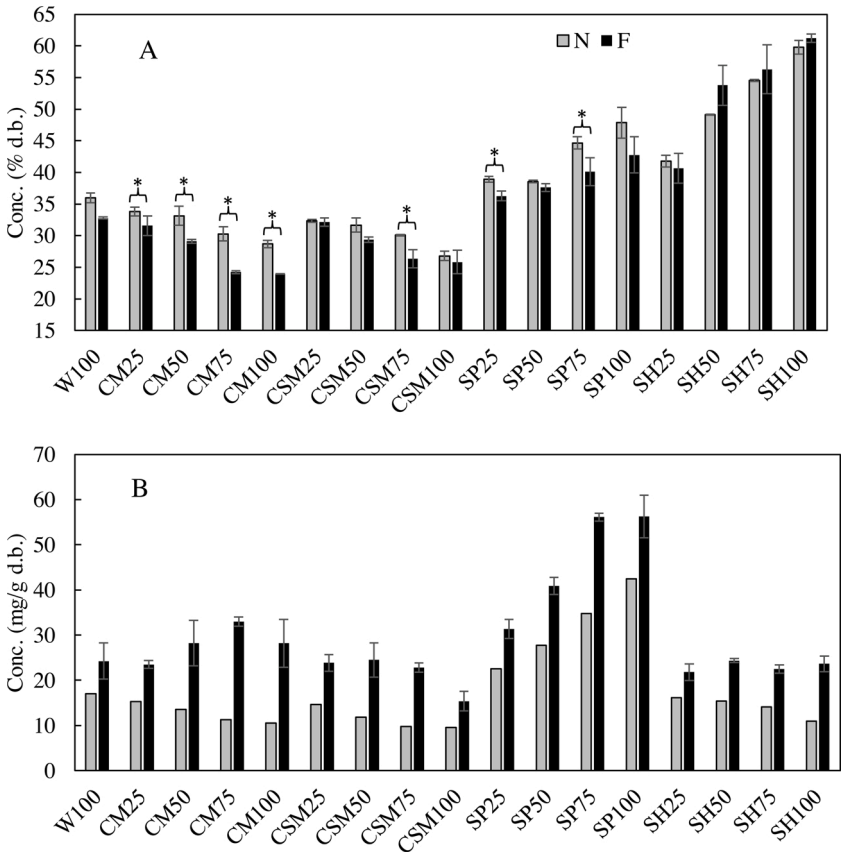

Fig. 4. Total structural carbohydrates (A) and reducing sugar (B) of WDGS mixing with canola meal (CM), cotton seed meal (CSM), sugar beet pulp (SP), and soybean hull (SH) at 0, 25, 50, 75 and 100 % mixing ratio at 0 day without inoculation, and after processing with *Rhizopus oryzae* for 4 days. Star mark represents significant ($p < 0.05$) reduction between treatments. N: non-fermented, F: fermented.

β-glucosidase, and xylanase [21]. The yield of reducing sugar was higher when higher mixing ratio of by-product was used. Fermenting substrate consisting of 75 % of CM resulted in reducing sugar increase by 1.92 fold, higher than 54 % increase in substrate with 25 % of CM. Similar trend of reducing sugar increase was also observed in substrate with increased mixing of CSM (from 63 % to 1.33 fold), SP (from 39 % to 61 %) and SH (from 34 % to 60 %) (Fig. 4B). It was noticed that 75 % mixing of CM, CSM, and SP showed the highest degradation of structural carbohydrates and increase of reducing sugar. Conversion of structural carbohydrates into reducing sugar could facilitate utilization of energy in the indigestible carbohydrates while improving palatability of the feeding ingredients for monogastric animals [49].

The changes of each component of structural carbohydrates before and after 4 days fermentation are shown in Table 3. Glucan made up of all cellulose and part of hemicellulose, and therefore is the major component of structural carbohydrates with proportion in the substrate

Table 3
Structure carbohydrate components in fermented and non-fermented substrate mixture by *R. oryzae*.

Substrate	Ratio	Glucan		Xylan		Galactan		Arabinan		Mannan	
		N*	F*	N	F	N	F	N	F	N	F
	% d.b.										
WDGS	100	**16.78**	**13.66**	7.80	7.29	1.93	2.90	6.86	6.75	2.61	2.21
WDGS/CM	75/25	**16.92**	**13.23**	6.37	7.76	2.36	2.23	6.24	6.40	1.94	1.95
	50/50	**16.22**	**11.78**	5.92	5.56	2.47	2.77	6.32	6.91	2.20	2.08
	25/75	**14.83**	**10.15**	3.59	3.80	**3.39**	**2.78**	6.21	6.11	**2.26**	**1.39**
	0/100	**14.15**	**11.37**	2.47	2.61	**3.92**	**3.23**	5.76	5.90	**2.38**	**0.84**
WDGS/CSM	75/25	**15.91**	**12.77**	6.97	7.20	2.19	2.64	5.63	7.06	1.65	2.50
	50/50	**15.50**	**12.03**	7.05	7.39	2.31	2.10	5.19	5.75	1.63	2.10
	25/75	**14.12**	**11.56**	7.65	6.95	**2.52**	**2.09**	4.24	4.36	**1.54**	**1.39**
	0/100	12.49	11.71	6.30	6.68	2.89	2.93	3.63	3.54	**1.50**	**0.98**
WDGS/SP	75/25	**19.55**	**14.51**	6.59	6.63	2.48	4.48	7.94	8.75	**2.34**	**1.90**
	50/50	**20.53**	**15.26**	4.58	4.70	3.03	5.77	9.16	10.35	1.24	1.51
	25/75	**23.58**	**17.18**	**3.45**	**2.71**	5.56	6.51	11.26	12.46	0.81	1.25
	0/100	**24.46**	**19.31**	**1.72**	**1.47**	7.33	6.98	14.35	14.99	0.00	0.00
WDGS/SH	75/25	21.67	20.97	7.91	7.61	2.19	3.06	6.44	6.38	**3.56**	**2.64**
	50/50	28.72	32.43	8.23	8.56	2.62	3.46	6.21	5.98	3.31	3.34
	25/75	33.68	36.63	8.03	7.94	2.78	3.44	**5.85**	**5.12**	**4.18**	**3.17**
	0/100	38.57	41.52	7.46	7.29	3.33	3.72	**5.56**	**4.99**	**4.86**	**3.72**

* N: non-fermented; F: fermented.

mixtures ranged from 10 to 40 % at dry basis. Fermentation by *R. oryzae* significantly ($p < 0.05$) degraded glucan by 18.6 % in WDGS, by 19–32 % in WDGS/CM mixture of all mixing ratio, by 18–23 % in WDGS/CSM mixture at 25, 50, and 75 % ratio, by 21−28% in WDGS/SP mixture of all mixing ratio. Suprisingly, WDGS/SH mixtures did not show reduction of glucan althrough they contain higher glucan content than all other mixtures. The glucan reduction of the substrates was primarily due to hydrolytic activity of cellulase and β-glucosidase produced by *R. oryzae* [21]. However, fermentation by *R. oryzae* did not have significant change in the fraction of xylan, galactan, arabinan and mannan in each substrate. The reduced fiber fraction in the fermented substrate would otherwise concentrate protein and total amino acids in the substrate (Fig. 1 and Fig. 3), thus improving digestible components in the fermented feed for monogastric animals [50]. On the other hand, the hydrolyzed products, mostly disaccharides and monosaccharides, from glucan can be beneficial for gut health in monogastric animals [49].

Highlighted value represent at least 10 % reduction of each component after fermentation.

3.4. Effects of fungal solid-state fermentation on degradation of phytic acid

Phytic acid or phytate is a principal storage form of phosphorous in plant-based feeding materials. It can form insoluble and less-digestible complex with protein, and reduce solubility of minerals by chelating effects especially with calcium [51]. The endogenous phytate-degrading enzymes in monogastric animals is incapable of hydrolyzing enough phytate-P, which usually leads to reduced growth and body weight caused by low digestibility of dietary protein and minerals [8]. Phytate concentration above 14 mg/g in the final diet formulation could have significant reduced digestibility of protein and amino acids in broiler chickens [52]. The concentration of phytic acid in CM (28.04 mg/g) and CSM (28.41 mg/g d.b.) was higher than it in WDGS (14.61 mg/g d.b.). Therefore, mixing CM or CSM with WDGS resulted in risk of higher level of phytic acid than WDGS alone. However, due to minimum phytic acid present in SP and SH, WDGS mixing with either SP and SH could dilute the phytate content in the substrate. Fermentation of 4 days by *R. oryzae* significantly ($p < 0.05$) decreased phytic acid by 54 % in WDGS, by 64 % in mixture with 25 % of CM, by 44 % in mixture with 50 % of CM (Fig. 5). The degradation was reduced when increasing mixture ratio of CM above 75 %. A significant reduction ($p < 0.05$) of phytic acid by 52 % and 28 % was also found in WDGS mixing with 25 % and 50 % of CSM, respectively. No significant reduction of phytic acid was found in increased mixing ratio of CSM above 75 %. In WDGS mixing with SP and SH, significant reduction ($p < 0.05$) of phytate (above 50 % reduction) was found in all mixing ratios except for 100 % SP and SH due to no existence of phytate. Phytase produced by *R. oryzae* played an important role in hydrolyzing phytic acid during fermentation [20]. The non-degraded phytate remained after fermentation could be due to the formation of phytate/protein complex through binding with certain amino acids on the protein surface, which could resist the access of phytase and therefore limit the hydrolytic activity [51]. Based on the results, it was suggested that WDGS mixing with lower ratio of CM or CSM (e.g. 25 % and 50 %) could achieve higher phytate degradation after *R. oryzae* fermentation. Mixing WDGS with SP and SH could dilute the high phytate content in WDGS, which could be further degraded to a lower level (below 5 mg/g) by *R. oryzae*.

R. oryzae as an edible fungal strain showed capability of reducing fiber content, degrading phytic acid, and enriching protein and amino acids in different substrate mixtures. CM, CSM, SP or SH alone is typically not used as feeding ingredient or inclusion ratio is lowered enough to reduce adverse effects on monogastric animals from either imbalanced amino acids, high fiber, high phytate content, or other anti-nutritional factors. Mixing these agro-industrial residues with WDGS provided chance for them to be modified to better fit nutrient requirement for monogastric animals. Generally, lower mixing ratio of the agro-industrial residue with WDGS showed better enrichment of protein and amino acid, and reduction of phytate, while higher mixing ratio is favorable for fiber degradation. In addition, protein-rich residues can facilitate protein and amino acids enrichment and fiber degradation but can be easy to degrade amino acids and accumulate ammonia. In comparison, mixing with fiber-rich residues results in less amino acids degradation and low phytate content after fermentation.

Solid-state fermentation of co- and by- products from corn-ethanol facility and other agro-industries can upcycle these materials together so as making them having potential or increased value for feeding purpose. Solid-state fermentation is a low-cost method due to its low water usage, low or zero effluent generation, and efficient for fungal growth. For monogastric animals who don't have rich rumen bacteria as in ruminants, the fermented feed should be low in fiber and anti-nutritional factors, high in protein (either from substrate or fungal strain), and the fungi should be safe for animal consumption. The goal of the process is to increase the inclusion ratio of the fermented feed in the monogastric animal diet, while maintaining or improving the feed intake and weight gain of the animals. This process can be easily adapted to the matured industrial mushroom producing facility with minimum modifications. However, the economic viability of this process depends on the final performance of animal feeding tests, market price of the fermented feed, and supply of the raw materials, which require further investigation.

4. Conclusion

Solid-state fermentation using *Rhizopus* strain provides chances of

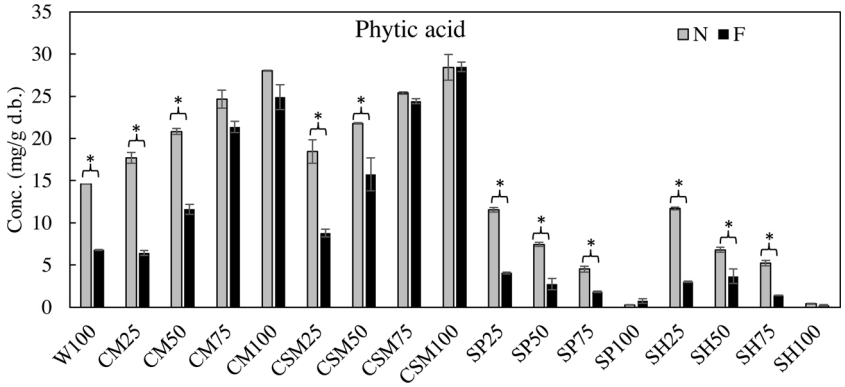

Fig. 5. Phytic acid concentration of WDGS mixing with canola meal (CM), cotton seed meal (CSM), sugar beet pulp (SP), and soybean hull (SH) at 0, 25, 50, 75 and 100 % mixing ratio at 0 day without inoculation, and after processing with *Rhizopus oryzae* for 4 days. Star mark represents significant ($p < 0.05$) reduction between treatments. N: non-fermented, F: fermented.

upgrading organic co-and by- products from different agro-industries to nutritive feeding ingredient for monogastric animals. Mixing WDGS with agro-industrial residues of different origins could potentially reduce the drying process, providing suitable substrate for fungal fermentation to improve overall feeding value. Four days fermentation of WDGS mixing with agro-residue showed potential for amino acids improvement with minimum ammonia accumulation. Ag-residue mixing ratio of less than 50 % resulted in higher phytate degradation but lower structural carbohydrates reduction. The substrate mixing with fiber-rich residues (mixture with C/N ratio higher than 3) could prevent loss of amino acids during 4 days of fungal processing. The study demonstrated the potential of combining WDGS with agro-residues in improving amino acid profile, reducing fiber and phytate by solid-state fermentation. The economic feasibility of this process require further study in animal feeding test and scale-up using existing industrial facilities.

CRediT authorship contribution statement

Xiao Sun: Investigation, Writing - original draft, Writing - review & editing, Data curation, Methodology, Formal analysis. **Yan Chen:** Investigation, Methodology. **Lina Luo:** Investigation, Methodology. **Fatemeh Heidari:** Investigation, Methodology. **Douglas G. Tiffany:** 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

No conflict of interest is applied to this manuscript.

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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.procbio.2021.10.029.

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