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RESEARCH PAPER

Enhanced protein and amino acids of corn–ethanol co-product by *Mucor indicus* and *Rhizopus oryzae*

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Abstract
Upcycle of co-products from corn–ethanol plant into protein-rich animal feed with balanced key amino acids via solid-state fermentation is a promising approach to economically support both biofuel and animal feed industries. However, there are multiple types of solid-state fermentation microorganisms and growth conditions that have not been tested. In this study, *Mucor indicus* and *Rhizopus oryzae* were used to ferment corn-based wet distiller's grains with solubles (WDGS). The effects of fermentation conditions (temperature, agitation, and moisture) and supplementations (extraneous carbon and nitrogen sources) were evaluated on protein production and amino acids profiles before and after fermentation. The study established best fermentation conditions (23 °C, static incubation for 4 days at 70% initial moisture content) to improve protein content for both *R. oryzae* and *M. indicus*. Moreover, urea supplied to *R. oryzae* and *M. indicus* improved protein concentration by 35 and 38%, and total amino acids content by 28 and 18%, respectively. The amount of 693.1 and 451.8 mg of additional total amino acids including 262.8 and 227.7 mg of key amino acids (lysine, methionine, tryptophan, and arginine) was synthesized by *R. oryzae* and *M. indicus*, respectively, per supply of 536 mg urea in 25 g of WDGS. This study demonstrated the feasibility of urea as a low-cost nitrogen source for amino acid biosynthesis in fungal fermentation of WDGS, which could contribute to the increasing demand for high-value monogastric animal feed.

Keywords Corn distiller's grains with solubles · Filamentous fungi · Amino acids · Urea · Solid-state fermentation

Introduction

As of 2019, United States has total of 190 operating ethanol biorefineries producing 16 billion gallons of fuel ethanol, contributing to 54% of the global fuel ethanol production [1]. Corn accounts for 95.8% of total feedstock used for ethanol production in US, and around 90% of the corn is converted via dry mill process to ethanol and by-products (e.g., distiller's grains, corn distiller oil). Over 37 million tons of distiller's dried grains with solubles (DDGS) were produced in 2019, which is used as an animal feed, predominantly for cattle (46% for beef cattle, 31% dairy cattle) but to a lesser extent, pigs (15%) and chickens (7%) [2]. Distiller's dried grains with solubles are widely utilized due to their low price (~ $150/ton) and relatively high protein content (~ 30%) [3]. However, DDGS have some drawbacks including an imbalanced amino acid profile because it contains several amino acids at a proportion lower than the requirement for pigs and poultry [4]. In addition, DDGS have relatively high content of indigestible fiber decreasing the efficiency of energy digestibility in comparison to cereal grains like corn [5]. These factors limit the use of DDGS feeding programs for nonruminants and lower its overall value as a feed commodity.

Increasing the feeding value of DDGS and other co-products from the corn ethanol stream (whole stillage, thin stillage, and wet distiller's grains) have been sought by researchers [6–8]. A common method used to improve nutrition, e.g. fats, protein profile, digestibility, and overall palpability, is to grow fungi in the co-product of interest. Filamentous fungi have been used to perform value added

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processes to corn ethanol co-products for the past decade [6, 7]. Patents had been granted on purification of thin stillage from dry-grind corn milling using fungi *Mucor indicus* and/or *Rhizopus microsporus* (*oligosporus*) [9]. These fungal strains are noted for their high protein content (~40%) and safety as a food additive. *Mucor* is high in chitosan, which has potential prebiotic properties in nonruminant animals while *Rhizopus* has a high content of essential amino acids containing approximately 7% of lysine [10]. *Aspergillus oryzae*, *Trichoderma reesei*, and *Phanerochaete chrysosporium* had been used to ferment soybean and corn processing co-products. Their studies demonstrated that a combination of fungal strains at different fermentation times for 6 days could aid to degrade up to 15% of fiber and increase protein content by 4.2%. These reductions in dietary fiber and increases in protein content are indicative that their process would be of benefit to animal feed [6]. *Aspergillus niger* NCIM 5613 has been used for solid-state fermentation of wheat bran and the culture conditions were optimized to generate high levels of phytase (38,500 IU kg^{-1} day^{-1}), xylanase (133.2 IU g^{-1} substrate), cellulase (41.58 IU g^{-1} substrate), and amylase (310.34 IU g^{-1} substrate). These enzymes are critical in biomass depolymerization [11]. Phytase produced during fungal processing of animal feed could be of additional benefit due to its catalytic ability to degrade phytate which is an indigestible phosphorous source for monogastric animals, such as chickens and pigs [12]. The production of fiber and starch degrading enzymes, such as cellulase, xylanase, and amylase is important in degrading polysaccharides into fermentable sugars for carbon source of fungal strains [13]. In a review paper by Ferreira et al. [14], the nutritional benefits of *Zygomycetes* fungi are outlined and heralded as a possible solution to production of chitosan which is a product of high commercial value and sustainable protein production [14]. *Rhizopus oligosporus* has comparable levels of lysine, tryptophan, and threonine to a traditional corn–soy diet for livestock but has significantly higher levels of methionine, which are often limited when attempting to incorporate nontraditional sources of animal feed. Besides amino acid profiles, lipid and fatty acids content also play important role in livestock nutrition. *Mucor circinelloides* was found to have a lipid content of 61% when grown in thin stillage as a substrate while also being a robust producer of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are nutritious long chain omega-3 polyunsaturated fatty acids [14].

Owing to their positive nutritional and growth characteristics, *Mucor indicus* and *Rhizopus oryzae* (two important *Zygomycetes* fungi) were selected as an attractive and suitable candidate and their potential in solid-state fermentation was explored in this present study. These fungi require appropriate moisture to grow and WDGS was selected due to its high moisture, economic infeasibility of transportation, and tendency to undergo spoilage rapidly as compared with DDGS. In addition, protein with high essential amino acids is critical for monogastric animal growth and performance. Therefore, this study evaluated the effects of substrate moisture content, incubation period, culturing temperature, and agitation on the protein content and amino acids profile of WDGS via solid-state fermentation using *M. indicus* and *R. oryzae*. Exogenous sources of carbon and nitrogen were explored to understand their impact on fungal growth, protein production, and amino acid profile in the solid-state fermentation system. This study would demonstrate feasibility of using fungal strains to improve protein and amino acids of corn–ethanol co-products as monogastric animal feed with potential to reduce feed cost for animal-raising farmers.

Materials and methods

Substrate

WDGS were obtained from a dry mill corn (*Zea mays*) ethanol plant (Absolute Energy, St. Ansgar, IA, USA) which follows a production that is representative of many US ethanol plants (Fig. 1). The sample of WDGS were aliquoted into 1 L polypropylene bottles and stored at −20 °C until use. Prior to use, the bottles were placed at 4 °C and allowed to thaw for 16 h. Moisture content of the WDGS was assessed by placing samples in a 105 °C oven overnight. The wet to dry weight ratio was used to calculate starting moisture content for experiments.

Microorganisms and maintenance of culture

Mucor indicus 24905 and *Rhizopus oryzae* was secured from the American Type Culture Collection (ATCC; Manassas, VA, USA). Both strains were prepared in the following way. Fungal spores were preserved at −70 °C in a 60% glycerol solution. Glycerol stocks were aseptically struck on potato dextrose agar plates and cultured at 30 °C for 7 days. To prepare the working spore solution, a sterile spreader was used to agitate the plates with sterile distilled water. The prepared store solution was placed in sterile 15 mL tubes. The stock spore solutions were used the same day they were prepared. To inoculate the fungal spores, a cell count of the spores was done using a 0.1 mm deep Neubauer improved hemocytometer (Hausser Scientific, Horsham, PA, USA) under microscope (National DC5-163 digital; National Optical and Scientific Instrument, Inc., Schertz, TX, USA) using 40× magnitude and Cellometer Auto X4 under Fluorescence (Nexcelom Bioscience LLC, Lawrance, MA, USA).

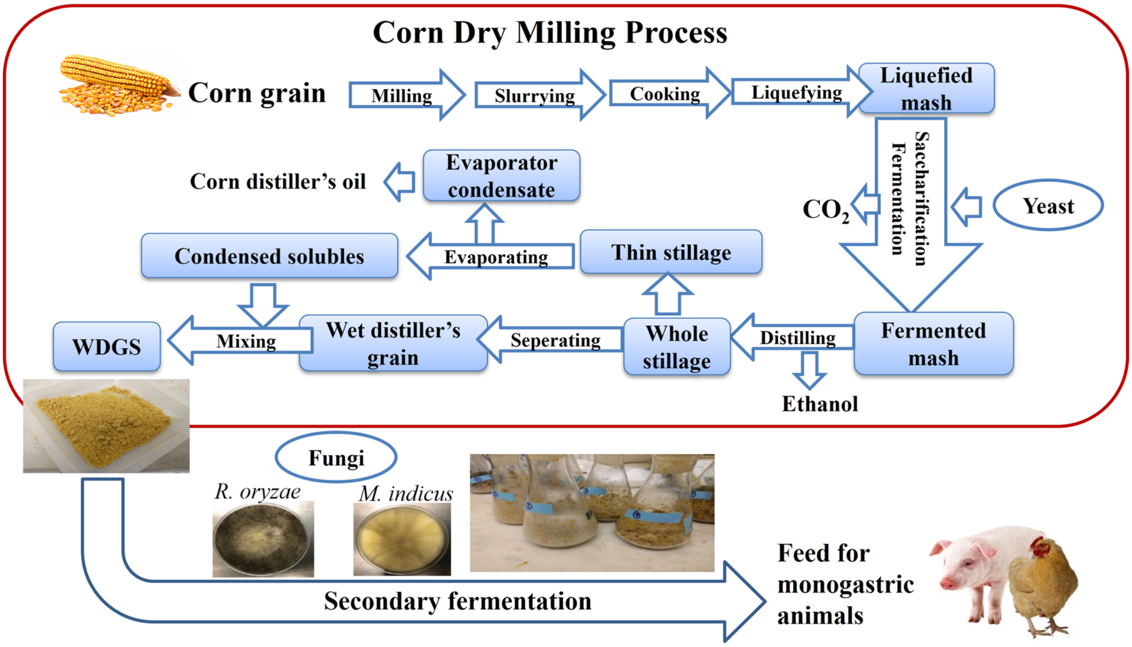

Fig. 1 Diagram of secondary fermentation of wet distiller's grains with solubles (WDGS) from corn–ethanol dry milling for improving nutritional value of WDGS as monogastric animal feed

Solid-state fermentation

A subsample (25 g) of WDGS was defrosted and placed in 250 mL Erlenmeyer flasks and autoclaved at 121 °C for 30 min and then cooled before use. 2.5×10^6 of *M. indicus* and *R. oryzae* spores per gram of wet substrate were inoculated to prepare separate monocultures (Fig. 1). The weight of each flask was measured after inoculation at 0 h and every 24 h of fermentation to monitor the loss of weight with time. Two fermentation flasks for each microorganism were harvested every 24 h and evaluated for protein production through time. Factors, such as moisture content, fermentation time, temperature, agitation, carbon and nitrogen supplementation, and different nitrogen species were studied by varying one factor at a time.

Effect of initial moisture content and fermentation time

Flasks were prepared according to "Solid-state fermentation" with moisture content being controlled through sterilized distilled water after autoclaving (121 °C for 30) and cooling. Moisture contents of 60 and 70% were evaluated. Fermentation was carried out for 4 days at 30 °C. Another set of flasks containing substrates with the moisture content adjusted at levels of 57, 61, 64, and 67% with sterilized distilled water were fermented for 14 days at 30 °C. 70% moisture content was then selected to evaluate the effect of shorter incubation time of 6 days on fermentation process.

Effects of fermentation temperature, agitation, supplementary of carbon and nitrogen source

Flasks were prepared according to "Solid-state fermentation". Solutions of 2.5 g of yeast extract and 2.5 g of glucose were prepared in distilled water and then passed through 0.45 µm filter. The prepared solutions were added to flasks enough to achieve the final moisture content to 70%. In flasks where only one nutrient solution was added, sterilized distilled water was used to bring the moisture content to 70% instead. Culturing temperature was evaluated at three different levels: 23, 30, and 37 °C. One treatment consisted of the culture flasks being hand-shaken vigorously once every 24 h for 30 s to dislodge substrate from the walls and encourage good mixing.

Effect of various nitrogen source on protein and amino acids enrichment

Flasks were prepared according to "Solid-state fermentation" with moisture content being controlled through sterilized distilled water after autoclaving and cooling. Different nitrogen compounds were evaluated. The chemical formula, nitrogen content amount added, and cost for each nitrogen compound are shown in Table 1. Urea (CH_4N_2O), sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$), ammonium nitrate (NH_4NO_3), ammonium chloride (NH_4Cl), and yeast extract were prepared in an aqueous solution and then sterile filtered through a 0.45 µm filter. Each solution was prepared

Table 1 Nitrogen content, application rate and cost of nitrogen compounds used in this study

Nitrogen compounds	Chemical formula	N content	Amount added (g)	Cost (\$)/kg[a]
Sodium nitrate	$NaNO_3$	16%	1.518	123.2
Sodium nitrite	$NaNO_2$	20%	1.232	100
Ammonium Chloride	NH_4Cl	26%	0.954	62.4
Ammonium nitrate	NH_4NO_3	35%	0.715	80.8
Urea	CH_4N_2O	47%	0.536	33.6
Yeast extract	–	10%	2.500	224

[a] The cost of each chemical was based on the price of it listed as ACS reagent (yeast extract was for microbial growth medium) in Sigma-Aldrich on October, 2020

to deliver 0.25 g of nitrogen and to bring the final moisture content to 70% in the flasks. The fermentation was performed for 6 days.

Analytical methods

Protein analysis

Protein fraction in each unfermented and fermented WDGS sample was determined by measuring the nitrogen content after subtracting ammonia nitrogen from the total nitrogen content using the Kjeldahl method [15] and then multiplied by 6.25. Briefly, oven dried (60 °C for 48 h) samples were completely digested in a solution containing sulfuric acid, potassium sulfate, and cupric sulfate at 420 °C for 45 min in DK20 Automatic Kjedahl Digestion Unit (VELP Scientifica, Inc., Bohemia, NY, USA). The digests were distilled in UDK129 Distillation Unit (VELP Scientifica, Inc., Bohemia, NY, USA) under high pH (achieved by 30% NaOH) to collect the released ammonia by absorption in 2% (w/v) boric acid solution with indicator (Methyl Red–Methylene Blue). The absorbed ammonia concentration was measured by titration using 0.02 mol L^{-1} HCl solution and converted to the total nitrogen and protein content. Weight loss of the fermentation mixture during fermentation was determined by subtracting the weight of each empty flask (predetermined before substrate preparation) with the total weight of the corresponding fermentation flask overtime. The moisture content of the fermentation sample was determined as percentage of weight loss after oven drying the sample at 105 °C overnight. The total solid content was calculated as the remaining percentage of the moisture content [16]. The total protein of each fermentation flask was determined by multiplying the protein fraction in dry weight of the fermentation mixture (measured weight multiplied by total dry matter).

Amino acid analysis

Selected samples were evaluated for their amino acid profile to better understand the effect of fermentation with addition of selected nitrogen sources on each amino acid. Prior to amino acid analysis, the dry solid samples were hydrolyzed to breakdown protein into amino acids. 50 mg of each sample was hydrolyzed with 1.0 mL of 0.6 mol L^{-1} HCl (hydrolysis for Trp was performed using 4.2 M NaOH) in 2 mL sealed centrifuge tube at 110 °C for 24 h. The headspace of each tube was purged with pure nitrogen before hydrolysis to avoid oxidation of certain amino acids (Met, Cys, and Trp). The hydrolyzed samples (sample hydrolyzed by 4.2 mol L^{-1} NaOH was neutralized with 0.3 mL of concentrated HCl) were diluted and filtered through 0.22 μm PTFE filter before quantification.

The amino acid analysis was performed in 1200 Infinity Series HPLC (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 3.5 μm) (Agilent Technologies, Inc., Santa Clara, CA, USA) 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) in place by HPLC auto sampler (G1329A, Agilent Technologies, Inc., Santa Clara, CA, USA) before injection [17]. Two mobile phases, A: 10 mol L^{-1} Na_2HPO_4, 10 mol L^{-1} $Na_2B_4O_7$, 5 mol L^{-1} NaN_3, pH 8.2, and B (by volume): acetonitrile: methanol: ultra-pure water 4.5: 4.5: 1, with different pumping ratios were used based on protocol [17]. A total of 21 amino acids in each sample were separated within the 40 min run at injection volume of 40 μL, column temperature of 40 °C and total flow rate of 1.5 mL min^{-1}.

Statistical analysis

The statistical analysis was performed with Tukey's multiple comparison of means at 95% confidence interval (P value < 0.05) using JMP Pro 15 (SAS Institute Inc., Cary, NC, USA). The statistical analysis was used to determine pairwise statistical differences ($P < 0.05$) of concentration and content of crude protein and amino acids between each treatment. The data in figures are presented as mean ± standard deviation except for evaluation of moisture content and fermentation time.

Results and discussion

Effect of initial moisture and fermentation time

The protein concentration and total protein content in each treatment of *R. oryzae* and *M. indicus* with varied moisture content and fermentation time were shown in Fig S1 as supplementary material. When compared with unfermented WDGS (WDGS 0d), total protein content after 4 days (4d) and 14 days (14d) fermentation at various moisture content generally showed not difference except for *R. oryzae* at 70% moisture content after 4 days fermentation (RO-70 4d). However, the protein concentration after 4 and 14 days fermentation showed improvement regardless of moisture content and strain species except for *M. indicus* at 67% moisture content after 14 days fermentation (MI-67 14d). The improved protein concentration with little change in total protein content could be due to the degradation of structural carbohydrates into volatile compounds in WDGS during fermentation, as *R. oryzae* and *M. indicus* had been reported to convert carbohydrates into alcohols and organic acids [18]. The average protein concentration after 4 days of fermentation regardless of strains and moisture content was 396 mg g^{-1}, which is higher than it after 14 days of fermentation (357.7 mg g^{-1}), indicating that 4 days is appropriate for improved protein concentration. A long fermentation time is typically not preferred for batch fermentation due to high production cost and low productivity. Some studies have indicated that their best fermentation time for protein enrichment was between 3 and 5 days [19–21]. Initial moisture content at 60% was better for *M. indicus* while 70% was preferred by *R. oryzae* for 4 days of fermentation. It was noticed that protein concentration and total content after 4 days of fermentation with *R. oryzae* at 70% MC increased by 35.4 and 23.4%, respectively, when compared with unfermented WDGS. This result was consistent with another study where the optimized protein improvement was obtained with white-rot fungi using 70% moisture content [22]. Therefore, the preferred fermentation time was 4 days for both *R. oryzae* and *M. indicus*. Higher initial moisture content (i.e. 70%) was suitable for *R. oryzae* while lower initial moisture content (i.e. 60%) was better for *M. indicus* in fermentation for protein improvement.

Effect of fermentation temperature, agitation, carbon and nitrogen supplementation

The protein fraction and total protein content as affected by supplementation of glucose and yeast extract, incubation temperature, and agitation for *R. oryzae* and *M. indicus* are featured in Fig. 2. When compared with unfermented WDGS, addition of glucose to *R. oryzae* fermentation (RO C, 30C, Static) didn't improve total protein content and protein fraction, while slight improvement of total protein by 11.1% was observed in *M. indicus* fermentation (MI C, 30C, Static), but the difference was not significant ($P > 0.05$). This could be due to limitation of available nitrogen source for protein accumulation. Supplementation of YE to WDGS improved total protein content by 11.4% when fermented by *R. oryzae* (Fig. 2a) and 22.5% when fermented with *M. indicus* (Fig. 2b). Fermentation by *R. oryzae* with addition of both YE and glucose and addition of only YE improved total protein content by 19.6 and 48.3%, respectively, as compared to fermentation with only glucose supplementation. This indicates that glucose present in substrate might compete with or inhibit the nitrogen source intake by *R. oryzae*. Therefore, reducing biomass accumulation. Glucose had also been demonstrated as not being preferred by *R. oryzae* in biomass and pellets formation as compared to potato dextrose [23]. In fermentation by *M. indicus*, addition of YE in presence of glucose also improved total protein by 21.7%. However, removal of glucose in presence of YE in fermentation by *M. indicus* didn't affect total protein content. It was

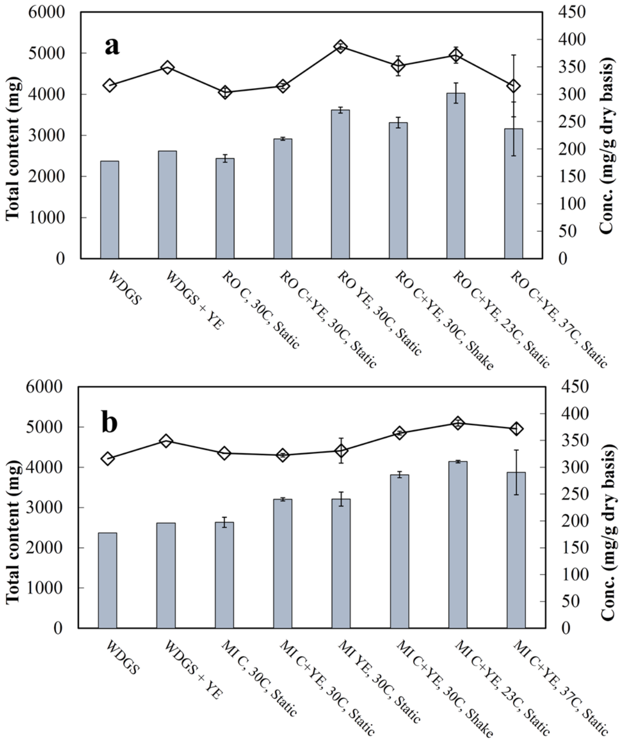

Fig. 2 Total protein content (gray bar) and concentration of wet distiller's dried grains with solubles (WDGS) at dry basis (open diamond) in different treatments of *R. oryzae* (**a**) and *M. indicus* (**b**) evaluating addition of carbon as glucose (C), nitrogen source as yeast extract (YE), incubation temperature (23°, 30°, and 37 °C), and agitation (static vs. shake)

concluded that for *R. oryzae* and *M. indicus*, glucose is not a necessary carbon source for improvement of protein synthesis and accumulation in the process of fermenting WDGS.

Agitation of fermentation bottles periodically slightly improved total protein content by 13.5% for *R. oryzae* and by 19.0% for *M. indicus*, but the differences are not statistically significant ($P > 0.05$). The slight improvement with agitation could be due to enhanced aeration in the substrate that facilitated aerobic activity for the fungal growth. However, agitation could also cause breakage of hyphae of filamentous fungi which adversely affects fungal growth [24]. Temperature is a critical parameter in fungal fermentation. The optimum temperature for growth, synthesis of enzymes, primary metabolites, and secondary metabolites are different. It was reported for *M. indicus* that the optimum temperature for production of oil, protein, and glucosamine was 28, 32, and 37 °C, respectively [25]. In the present study, the highest total protein production was achieved at 23 °C for both *R. oryzae* (38.2 and 27.6% higher than at 30 and 37 °C, respectively) and *M. indicus* (29.2 and 6.9% higher than 30 and 37 °C, respectively). Incubating temperature for fungi is typically lower than that for bacteria, as reported [19] using 28 °C for *Aspergillus oryzae* and 37 °C for *Bacillus subtilis*. Statistical analysis showed no significant ($P > 0.05$) difference was observed for protein fraction in all the treatments with both *R. oryzae* and *M. indicus*. However, *R. oryzae* and *M. indicus* with supplementation of glucose, YE and was incubated at 23 °C statically significantly ($P < 0.05$) improved total protein content by 69.9 and 74.7%, respectively. It was concluded that YE, which provided crude protein equivalent to 10% of the total crude protein in WDGS, was effective nitrogen source in improvement of total protein content for both *R. oryzae* and *M. indicus* while glucose was not necessary. Besides, intermittent agitation and lower incubating temperature (23 °C) were preferred for both *R. oryzae* and *M. indicus* in protein improvement.

Effect of various nitrogen source

The effects of different extraneous nitrogen sources on protein content and fraction in WDGS fermentation by *R. oryzae* and *M. indicus* were depicted in Fig. 3. Among all the nitrogen compounds, urea (CH_4N_2O) and YE are organic compounds, while sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$), ammonium chloride (NH_4Cl), and ammonium nitrate (NH_4NO_3) are inorganic compounds. There is no clear advantage of organic or inorganic nitrogen compounds over the other in improving total protein and protein fraction for *R. oryzae* and *M. indicus*. In fermentation by *R. oryzae*, addition of YE resulted in the highest protein fraction followed by urea (Fig. 3a). The results of fermentation by *R. oryzae* were consistent with another study where addition of YE improved fungal biomass of *R. oryzae* more than addition of urea [26]. Fermentation of WDGS by *R. oryzae* with YE supplementation improved total protein content by 22.9% and protein fraction by 14.0% as compared to unfermented substrate (Fig. 3a). YE was considered beneficial in promoting cell growth due to its rich nutrient contents (minerals, amino acids, vitamins and growing factors) while urea was advantageous in certain metabolite production [26]. YE had also been used as suitable nitrogen source in chitosan production by *M. indicus* [27]. Supplementation of urea resulted in the highest protein fraction in fermentation by *M. indicus* while the total protein content was similar as that supplemented by YE (Fig. 3b). Urea also had been reported to greatly improve mycelium protein of *R. oryzae* while it has less impact on the amino acid profile of biomass [28]. However, considering the cost of YE and urea (Table 1), urea would be preferred as a nitrogen source in large scale production.

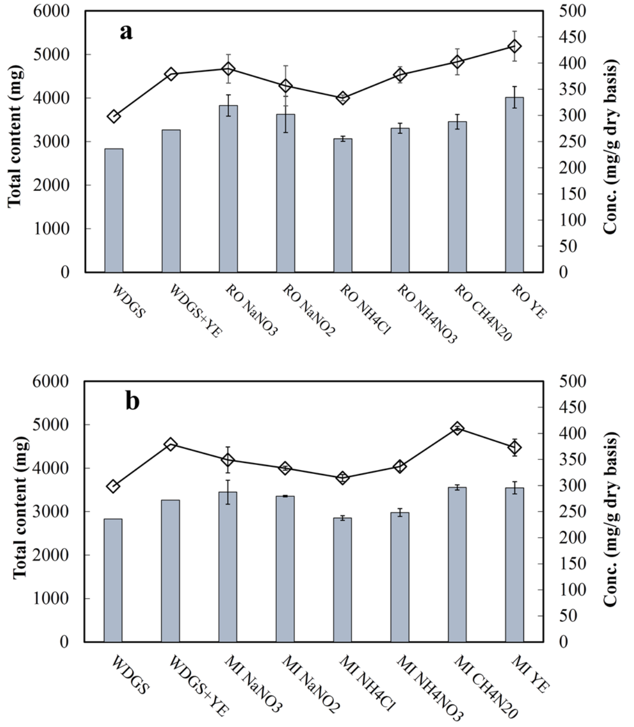

Fig. 3 Total protein content (gray bar) and concentration (open diamond) in different treatments of *R. oryzae* (**a**) and *M. indicus* (**b**) evaluating effect of different nitrogen source: sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$), ammonium chloride (NH_4C_l), ammonium nitrate ($NaNO_3$), urea (CH_4N_2O), yeast extract (YE)

The nitrogen supplemented via each compound was the same (1.0% dry basis of substrate). Therefore, the treatment WDGS + YE can be used as a baseline for unfermented WDGS as compared to supplementation with other nitrogen compounds. It was noted that fermenting WDGS with ammonium nitrogen, such as NH_4Cl and NH_4NO_3 did not improve either total protein content or protein fraction for both *R. oryzae* and *M. indicus* as compared to the same substrate without fermentation. The addition of NH_4Cl resulted in declined total content and concentration of crude protein after fermenting by *M. indicus* and *R. oryzae*, indicating that NH_4Cl could be an inhibitory compound for growth of *R. oryzae* and *M. indicus*. However, ammonium nitrogen in the form of ammonium sulfate ($(NH_4)_2SO_4$) when supplemented improved protein production in brewery spent grain fermented by *Rhizopus oligosporus* [29] and yam peel waste fermented by *Saccharomyces cerevisiae* [21]. Supplementation of $NaNO_3$ and $NaNO_2$ improved total protein content of *R. oryzae* fermented WDG by 17.1 and 10.9%, respectively. Not much improvement of total protein content was found in *M. indicus* fermented WDGS with supplementation of $NaNO_3$ and $NaNO_2$. However, supplementing WDGS with $NaNO_2$ reduced the protein fraction for both *M. indicus* and *R. oryzae* when compared with unfermented substrate.

From statistical point of view, the differences of the impacts by adding all the nitrogen compounds ($NaNO_2$, $NaNO_3$, NH_4Cl, NH_4NO_3, urea, and YE) for *M. indicus* and *R. oryzae* in total protein and protein concentration were not significant ($P > 0.05$). However, due to price advantage and availability, urea could be a potential nitrogen source for scaled protein improvement by fermentation of WDGS with *M. indicus* and *R. oryzae*.

The concentration and total content of crude protein and amino acids in fermentation treatments with the addition of selected nitrogen source YE, $NaNO_{3,}$ and urea (CH_4N_{20}) by *R. oryzae* and *M. indicus* and nonfermented substrate WDGS with YE (WDGS + YE) and WDGS only (WDGS) were shown in Table 2. Amino acids accounted for 86.9% of total crude protein in WDGS. The addition of YE to WDGS improved concentrations of total crude protein and total AA by 27.2% and 36.3%, respectively, indicating that YE is a nitrogen source rich of amino acids. The total CP content in the fermentation treatments with the addition of nitrogen compounds did not have significant difference ($P > 0.05$) as discussed in the previous section due to the total nitrogen of the substrate was the same. The increased CP concentration in WDGS + urea fermented by *M. indicus* and WDGS + YE or urea fermented by *R. oryzae* (Table 2), although not significant ($P > 0.05$), could be due to reduction in other components, such as fiber and lipid in the substrate that were consumed by the fungal strains. The total AA concentration and content in the substrate WDGS + YE and its fermented product by *M. indicus* and *R. oryzae* had no significant differences ($P > 0.05$). Owing to rich AA content in YE, fermentation of WDGS + YE might simply transform AA in the substrate into AA of the fungal biomass without net change of total AA. However, WDGS with urea after fermentation by *M. indicus* and *R. oryzae* showed significantly higher ($P > 0.05$) total AA concentration than nonfermented WDGS (Table 2). This indicates that the extraneously added urea had been used by both fungi for AA synthesis. It was observed that a quantity of 536 mg of urea (containing 250 mg of N) added to 25 g of WDGS was converted into around 367 and 589 mg of AA by *M. indicus* and *R. oryzae*, respectively. Supplement of $NaNO_3$ to WDGS did not show improvement of either total AA content or concentration by *M. indicus* and *R. oryzae*, suggesting that $NaNO_3$ may not be an efficient nitrogen source for *M. indicus* and *R. oryzae* to accumulate AA.

The essential and limiting amino acids in diets of monogastric animals (swine and poultry) include isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), threonine (Thr), tryptophan (Try), and valine (Val). The changes of these AA after fermentation by *M. indicus* and *R. oryzae* with supplementation each of YE, $NaNO_3$, and

Table 2 Total CP and AA concentration and content in nonfermented and fermented WDGS with supplement of selected nitrogen source

Substrate or product[a]	WDGS	WDGS + YE[d]	WDGS + YE	WDGS + $NaNO_3$	WDGS + urea	WDGS + YE	WDGS + $NaNO_3$	WDGS + urea
Strain	–	–	MI	MI	MI	RO	RO	RO
CP concentration[b] (mg g^{-1})	298	379	373.0 ± 16.2	349.2 ± 24.9	409.7 ± 3.7	432.5 ± 28.6	389.2 ± 27.5	402.3 ± 24.8
CP content[c] (mg)	2833	3267	3549 ± 141	3448 ± 275	3556 ± 60	4014 ± 246	3826 ± 245	3455 ± 169
AA concentration (mg g^{-1})	261.3	360.5	320.1 ± 12.7	200.8 ± 13.8	337.9 ± 27.1	340.5 ± 2.02	202.9 ± 11.4	369.4 ± 1.04
AA content (mg)	2482	3107.4	3046.3 ± 101	1982.9 ± 160	2933.8 ± 267	3161.4 ± 3.2	1995.5 ± 93.7	3175.1 ± 66.5

[a] WDGS: wet distiller's grain and solubles, YE: yeast extract, MI: *Mucor indicus*, RO: *Rizopus oryzae*, The average dry weight of substrate in flask without fermentation was: WDGS 9.5 g, WDGS + YE 8.62 g; the average dry weights of substrate before and after fermentation were: WDGS + YE MI 10.9 and 9.5 g, WDGS + $NaNO_3$ MI 10.6 and 9.9 g, WDGS + CH_4N_2O MI 10.5 and 8.7 g, WDGS + YE RO 10.8 and 9.3 g, WDGS + $NaNO_3$ MI 10.8 and 9.8 g, WDGS + CH_4N_2O MI 10.6 and 8.6 g

[b] CP: crude protein, AA: amino acids, CP or AA concentration represents the mass of CP or AA per gram of dried substrate or product

[c] CP or AA content represents the total mass of CP or AA in fermentation flask (calculated by multiplying its concentration by the mass of dried substrate or product in flask)

urea were shown in Fig. 4. The content of Ile, Leu, Thr, and Val in nonfermented WDGS, WDGS + YE, and fermented WDGS + YE, WDGS + urea by *M. indicus* and *R. oryzae* had no significant differences ($P > 0.05$), indicating that these four amino acids can neither be provided by YE nor synthesized by *M. indicus* and *R. oryzae*. The content of Arg, Met, Trp, and Lys were significantly improved ($P < 0.05$) after fermentation by both fungal strains when

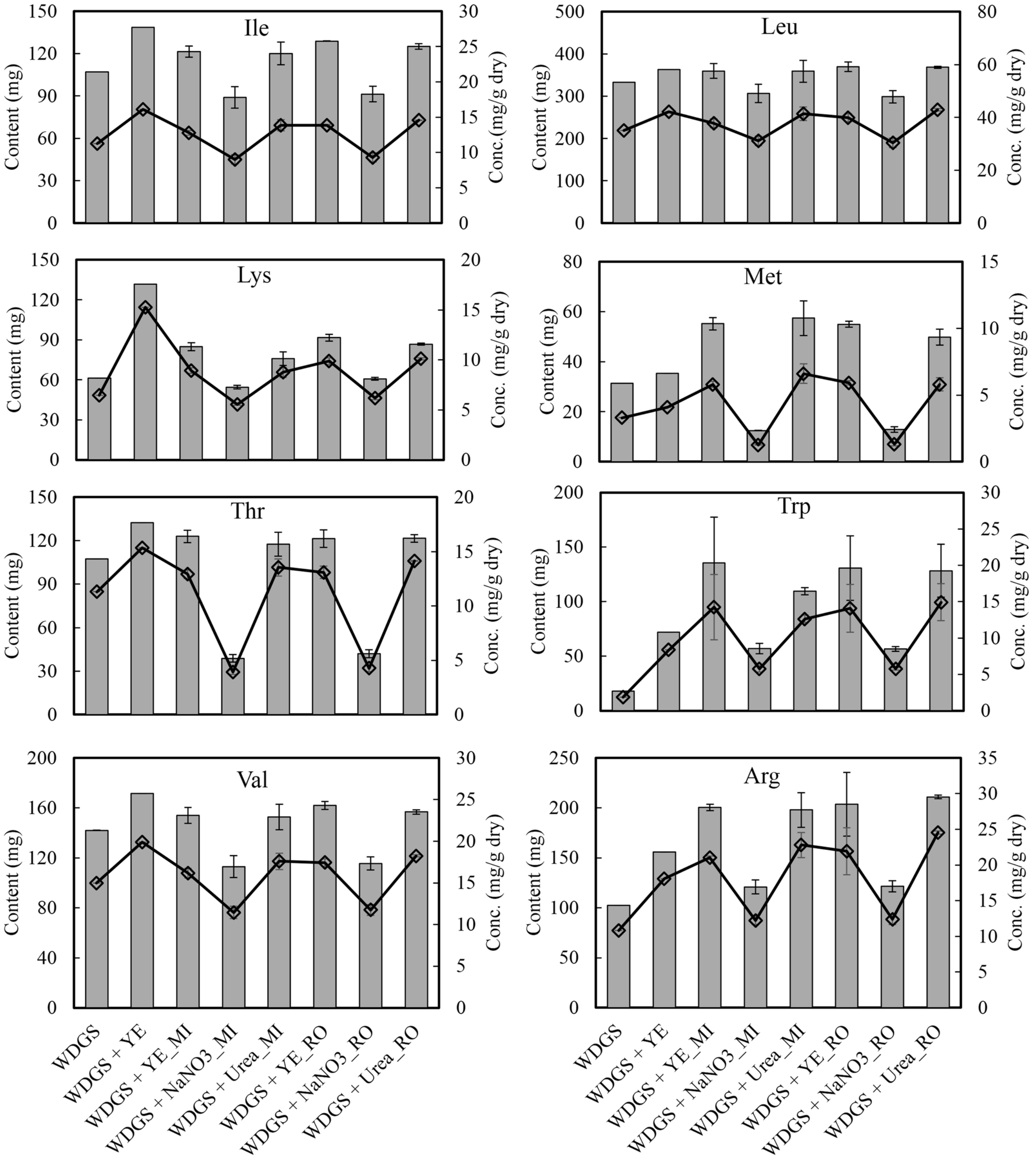

Fig. 4 Concentration (open diamond) and content (gray bar) of Ile, Leu, Lys, Met, Try, Thr, Arg, and Val in treatment using *R. oryzae* (RO) and *M. indicus* (MI) with addition of YE, $NaNO_3$, and urea and non-fermented substrate WDGS, and WDGS + YE. Error bar represents standard deviation of two replications

WDGS was supplemented with urea. It was noticed that 95.4 and 108.4 mg of Arg, 26.1 and 18.5 mg of Met, 91.49 and 110.4 mg of Trp, 14.7 and 25.6 mg of Lys were synthesized by *M. indicus* and *R. oryzae*, respectively, when 536 mg of urea (250 mg of N) was added. The total amount of Arg, Met, Trp, and Lys converted by *M. indicus* and *R. oryzae* from urea was 227.7 and 262.9 mg, respectively.

The concentrations of Arg, Val, Met, Trp, Ile, and Lys in WDGS with the addition of urea were significantly enhanced ($P < 0.05$) after fermentation by *M. indicus* and *R. oryzae* (Fig. 4). Among these amino acids, however, the Ile and Val didn't change much in their content as discussed above, which indicates that these two AA were concentrated as a result of weight loss of substrate during fermentation. The other amino acids Arg, Met, Trp, and Lys were concentrated due to both the conversion of urea and weight loss of substrate during fermentation. In treatments of WDGS with $NaNO_3$ addition, the concentrations of Thr, Val, Met, and Trp were significantly reduced ($P < 0.05$) through either consumption as nitrogen source or converting to other AA by the fungal strains. The other amino acids Arg, Ile, Lue, and Lys didn't have significant ($P > 0.05$) change in concentration after fermentation by either *M. indicus* or *R. oryzae* as compared to WDGS without fermentation. Therefore, $NaNO_3$ may not be an efficient nitrogen source for production of the limiting amino acids for monogastric animal diets.

Urea as a low-cost organic nitrogen source has shown its advantage in WDGS fermentation by both *M. indicus* and *R. oryzae* (Table 2; Fig. 4). Total AA content increased by 18.2 and 27.9% in *M. indicus* and *R. oryzae* fermented WDGS, respectively (Table 2). Significant improvement ($P < 0.05$) of the total AA content was found in Arg (by 105.7%), Met (by 59.0%), Lys (by 41.7%), and Trp (by 7.15-fold) when fermenting WDGS supplemented with urea by *R. oryzae* (Fig. 4). In *M. indicus* fermented WDGS with urea, the content of Arg, Met, Lys and Trp was enhanced by 93.0, 83.2, 24.0%, and 6.1-fold, respectively (Fig. 4). This demonstrated that the two fungal strains *R. oryzae* and *M. indicus* could use urea for key amino acids synthesis in its own biomass. This improvement in amino acid content and concentration can be of significant nutritional value to poultry and swine feeding programs because Arg, Met, Lys, and Trp are essential amino acids of which supply by common feed ingredients is relatively low [30–32].

The concentration of each and total amino acids in total CP varied in different treatments as shown in Table 3. YE had high fraction of AA and increased the AA/CP ratio when supplemented to WDGS. However, fermentation of WDGS + YE substrate by both fungal strains reduced the total AA/CP ratio, indicating that the fungal strains

Table 3 Ratio of total amino acids (AA) content to total crude protein (CP) content in non-fermented and fermented WDGS with supplement of selected nitrogen source

Substrate	WDGS	WDGS + YE	WDGS + YE	WDGS + $NaNO_3$	WDGS + urea	WDGS + YE	WDGS + $NaNO_3$	WDGS + urea
Strain			MI	MI	MI	RO	RO	RO
Fraction of AA in total CP (%)								
Asp	5.76	6.31	5.45	4.60	4.63	4.92	4.26	5.70
Glu	18.60	17.15	16.90	11.50	16.44	15.50	9.88	19.47
Ser	4.75	4.95	4.27	2.38	4.16	3.48	2.24	4.52
His	2.73	2.69	2.32	1.75	2.32	2.04	1.55	2.38
Gly	3.79	4.00	3.26	2.40	3.22	2.95	2.23	3.58
Thr	3.79	4.05	3.47	1.13	3.30	3.03	1.10	3.52
Arg	3.62	4.76	5.64	3.50	5.57	5.07	3.18	6.11
Ala	7.29	7.55	7.22	4.48	7.12	7.17	4.06	7.81
Tyr	2.38	2.17	2.12	0.53	2.13	2.13	0.22	2.20
Cys	0.32	0.33	0.39	0.24	0.45	0.29	0.21	0.40
Val	5.02	5.25	4.34	3.28	4.29	4.03	3.02	4.54
Met	1.11	1.08	1.56	0.36	1.61	1.37	0.34	1.44
Trp	0.63	2.21	3.82	1.65	3.08	3.26	1.48	3.71
Phe	4.78	4.85	4.39	3.36	4.16	4.02	3.05	4.44
Ile	3.78	4.25	3.42	2.58	3.38	3.21	2.39	3.62
Leu	11.75	11.10	10.14	8.90	10.10	9.20	7.81	10.66
Lys	2.16	4.03	2.39	1.58	2.14	2.28	1.59	2.51
Hyp	5.36	8.37	4.75	3.28	4.40	4.79	3.55	5.28
Total	87.62	95.12	85.85	57.51	82.49	78.75	52.15	91.90

Table 4 Protein improvement by solid-state fermentation of agro-industrial waste using bacteria and fungi

Biocatalysts	Substrate	Fermentation condition	Crude protein (CP) and amino acids (AA) enrichment (in dry matter)	References
Bacillus subtilis	Cassava pulp/corn grain mixture	14 days, supplemented with 2% urea, 1.5% diammonium phosphate	CP enriched by 11.1%	[34]
Rhizopus oligosporus	Brewery spent grain	Supplemented with $(NH_4)_2SO_4$, urea, and $NaNO_3$	CP enriched by 46.1% (without N supplement), by 83.2% ($(NH_4)_2SO_4$), by 78.7% (urea), by 52.6% ($NaNO_3$)	[29]
Trichoderma reesei	Peels from mango, apple, banana, orange, tomato	5 days at 28 °C	CP enriched by 37.3% (mango peel), by 22.5% (apple peel), by 48% (banana peel), by 235% (tomato peel)	[20]
Saccharomyces cerevisiae	Yam peal	4 days, supplemented with and without $(NH_4)_2SO_4$	CP enriched by 67.9% without $(NH_4)_2SO_4$ and by 135% with $(NH_4)_2SO_4$, total AA increased by 3.8 fold	[21]
Co-culture of *Aspergillus oryzae* and *Bacillus subtilis*	Mixture of sweet potato beverage residues and peanut shells	3 days at 30 °C	CP enriched by 48.4%	[19]
Aspergillus oryzae	Soy meal	36 h	CP enriched by 16.9%, total AA improved by 13%	[35]
Co-culture of *Saccharomyces cerevisiae*, *Lactobacillus delbruckii*, and *Lactobacillus coryneformis*	Cassava pulp	7 days	Protein content enriched by 162%	[33]
Candida utilis, *Saccharomyces cerevisiae* and their co-culture	Cassava root	4 days	CP enriched by 19.75 fold (*Candida utilis*), 15.85 fold (*Saccharomyces cerevisiae*) and by 25 fold (co-culture)	[36]
Aspergillus fumigatus, *A. flavus*, *A. niger* and *Trichoderma* sp.	Cassava peel waste	72 h	CP enriched by 13 fold (*A. fumigatus*), by 9.65 fold (*A. flavus*), by 8.69 fold (*A. niger*) and by 17.6 fold (*Trichoderma* sp.)	[37]
Schizophyllum commune, *Phanerochaete chrysosporium*	Mixture of banana, pineapple, and papaya peels	7 days at 32 °C, moisture content 70%, pH 5.4 and inoculum size 6.1%	Maximum protein reached 198.77 mg g^{-1}	[22]
Rhyzopus oryzae, Mucor indicus	WDGS	6 days at 23 °C, supplemented with urea	CP concentration enriched by 35% and 38% for *R. oryzae* and *M. indicus*, total AA content improved by 28% and 18% for *R. oryzae* and *M. indicus*	This study

consumed AA in the substrate during fermentation. $NaNO_3$ is an inorganic nitrogen source which contributed to total CP but could not be used as efficient nitrogen source for AA synthesis by both fungi. In addition, the two fungal strains also consumed AA in WDGS, which resulted in remarkable reduction of total AA/CP ratio (Table 3). The ratio of total AA/CP in WDGS + urea after fermentation by *M. indicus* didn't increase as compared to it in WDGS. Urea as organic nitrogen compound (47% N) greatly contributed to total CP (N × 6.25), which could reduce total AA/CP ratio if no or lower net AA was synthesized. In fact, urea contains 2.13 times higher of N than it in the CP of WDGS. Therefore, the increase of total AA/CP ratio should be observed if more than 34% of N in the added urea was converted into AA. Although total concentration and content of AA were improved in WDGS + urea by *M. indicus*, the conversion efficiency of N in urea into total AA was lower than 34%. However, the ratio of total AA/CP in WDGS + urea by fermentation of *R. oryzae* was higher than that in WDGS, indicating that the conversion efficiency of N in urea into AA by *R. oryzae* was higher than 34%. Compared with WDGS, *R. oryzae* fermented WDGS + urea had higher fraction of Glu, Arg, Ala, Cys, Met, Trp, and Lys, while *M. indicus* fermented WDGS + urea had higher fraction of Cys, Met, and Trp. This improved AA fraction could be originated from synthesis of fungal biomass.

Protein and amino acids are important nutrients for monogastric animal feed and their improvement have been studied using SSF on numerous agro-industrial residues (Table 4). Different types of microorganisms have been tested in both pure culture and mixed culture for bacteria and fungi. Bacteria, such as *Bacillus subtilis*, *Lactobacillus delbruckii*, *Lactobacillus coryneformis,* and fungal strains, such as *Aspergillus niger*, *A. oryzae*, *A. fumigatus*, *A. flavus*, *Trichoderma* reesei, and yeast, such as *Saccharomyces cerevisiae* had been demonstrated to enrich protein content and amino acids by processing of agro-processing wastes and fruit wastes. Besides pure culture fermentation, co-culture of *Aspergillus oryzae* and *Bacillus subtilis* [19], *Saccharomyces cerevisiae*, *Lactobacillus delbruckii*, and *Lactobacillus coryneformis* [33] had also been reported and sometimes more preferable to pure culture in protein enrichment. Bacteria, due to their low doubling time and efficient energy utilization, are also preferable to fungi in some cases as reported in a study fermenting cassava pulp and corn grain mixture using *Bacillus subtilis* (crude protein increased by 11.1%, true protein by 59.6%) and *Aspergillus niger* (crude protein by 4.9% and true protein by 22.1%) [34]. Additional nitrogen source (ammonium sulphate) supplementation also assisted protein and amino acids enrichment as reported that 4 days fermentation increased protein content in yam peal from 66.0 to 155.4 mg g^{-1} and total amino acids by 3.8 fold with addition of nitrogen source while the protein content increased to 110.8 mg g^{-1} without adding nitrogen source [21]. Using bacteria strains and co-culture fermentation of WDGS would further improve protein content while enriching essential amino acids content and reducing indigestible fiber, which deserves future research. Urea has long been used as replacement of vegetable and animal protein source in ruminant diets due to the presence of rumen ureolytic bacteria in the gastrointestinal tracts of the ruminants. These bacteria hydrolyze urea into ammonia and then microbial protein and amino acids for ruminant nutrition. The present study demonstrated that two fungal strains (*R. oryzae* and *M. indicus*) fermenting WDGS at solid state could convert the supplemented urea into amino acids that are critical for monogastric animal nutrition, providing a solution of indirectly using urea as feed for monogastric animal. Fungi of different type have preference of different nitrogen source, therefore, more fungal strains and their favorable exogenous nitrogen source need to be discovered. The effects of possible unspent exogenous nitrogen in the diet on monogastric animal worth further evaluation to fully justify the feasibility of this process for production of monogastric animal feeding ingredients.

Conclusions

Corn-based WDGS can serve as an effective substrate for fungal growth under controlled conditions. Supply of urea significantly improved total amino acids concentration by 41 and 29% in *R. oryzae* and *M. indicus*, respectively. Greatly improved among them are Ile, Lys, Met, Trp, and Arg which are critical amino acids for monogastric animal nutrition. This study established the best conditions for fungal fermentation and uncovered the feasibility of urea as a low-cost nitrogen source for protein and amino acids enrichment by solid-state fermentation, which could contribute to both corn–ethanol and animal feed industries.

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Declarations

Conflict of interest The authors declare that no conflict of interest is applied to the work reported in this manuscript.

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