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PHYTIC ACID CONTENT AND DIGESTIBILITY OF COCONUT RESIDUES DERIVED-PROTEINS AFTER SOLID-STATE FERMENTATION BY *Aspergillus awamori*

AIZAT MOHD-RAZALI[1], MASDUKI MOHAMAD MORNI[2], MARIAM TAIB[3] and AZIZ AHMAD[2,3*]

[1]*Faculty of Engineering Science and Technology, Infrastructure University of Kuala Lumpur, 43000 Kajang, Selangor, Malaysia*
[2]*Centre for Fundamental and Continuing Education, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*
[3]*Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*
[*]*E-mail: aaziz@umt.edu.my*

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ABSTRACT

The growing industry of aquaculture is in demand of alternative protein sources as fish feeds. An adequate amount of digestible protein and the presence of anti-nutritional such as phytic acid factors are limiting factors in feed formulations. One alternative source of protein for fish feeds is fermented coconut residues. However, the phytic acid content, an anti-nutrient compound produced during the bioconversion or fermentation process of agriculture waste has not yet been determined. Therefore, the objective of the present study was to determine the phytic acid content and protein digestibility of coconut residues after solid-state fermentation (SSF) by *Aspergillus awamori*. Samples from three optimized fermentation conditions were analyzed for soluble proteins, phytic acid contents, and in-vitro digestibility activity by trypsin and pepsin. Results showed that phytic acid content in all samples were lower than 0.1 mg/g dry wt. of the sample after seven days of fermentation. The protein digestibility by trypsin (ranging from 36.54 ± 4.22 to 43.22 ± 2.46%) was higher than pepsin, where it fell within the percentage required for fish feed formulation. The findings suggested that fermented coconut residues are a highly potential alternative source of protein for fish feed formulation.

Key words: Fish feed, protein, phytic acid, *in vitro* protein digestibility

INTRODUCTION

The expansion of the aquaculture industry especially in Asia has induced tremendous demand for aqua-feed, where the production rate has exceeded 30% annually. The cost of feed could rise to 60% of the total cost for industry and the demand is continuous. Proteins appeared as the most expensive nutrients in feed, which subsequently caused increasing costs in feed production, growth of fishes, industrial maintenance, and finally the price of fishes (Gaylord & Barrows *et al.*, 2009). The protein requirement is species-specific with regards to herbivorous, omnivorous, and carnivorous fishes. To compute an adequate amount of protein in feed, the content of essential amino acids must meet the requirements of the species to be fed. Alternative protein sources are strongly recommended to sustain the growing industry of aquaculture to reduce the cost of feeding. Protein in feed depends on its availability, digestibility, the content of amino acids and nutrients, anti-nutritional factors, and significantly the cost of production and species (El-Sayed *et al.*, 1999; Gilani *et al.*, 2005; Abdelnour *et al*., 2018).

Over the years, plant-derived feeds such as palm kernel cake and coconut residues (Syahri *et al.*, 2016; Farizaldi *et al.*, 2017), pineapple pulp and cassava (de Lima *et al.*, 2012; Duy & Khang, 2017) have been introduced to minimize the cost of feeds. The recycling of the waste matters was also proven to be useful in reducing the production cost

* To whom correspondence should be addressed.

up to 20% off the feed and feeding cost for the industry. The uses of microbes particularly fungi in converting plant carbohydrates as a substrate with supplementation of salt to protein is a useful approach (Ramachandran *et al*., 2004). Coconut residues in general contain soluble sugar, protein, starch, lipids, and traces of nitrogen (Ramachandran *et al*., 2004). The high amount of carbohydrate fiber instead of protein (Francis *et al*., 2001) has triggered many studies to improve its nutritional value. Additionally, fermented copra meals have been tested on grouper (Mamauag *et al.*, 2019). The fungus *Aspergillus* is known as one of the fungi most commonly used in improving the protein content of cassava and sugar cane bagasse (Moo-Young *et al*., 2000). *Aspergillus awamori* is commonly known for the *awamori* process, which is the conversion of starch to citric acid. During the fermentation process, the starch digestion enzymes are among the type of soluble proteins that are secreted onto the substrate. These secreted proteins normally identified as amylase and starch-disbranching enzymes, have been utilized in the diet of broiler chickens (Morgado *et al.*, 2016) and laying hens (Saleh *et al.*, 2017). In our previous study, the optimum condition for solid-state fermentation of coconut residues by *A. awamori* has been designated (Mohd-Razali *et al.*, 2019). Nonetheless, the accumulation of anti-nutrient content such as phytic acid and protein digestibility, which is crucial in feed formulation has not been reported. Therefore, the objective of the current study was to determine the phytic acid and soluble protein contents of coconut residues after selected fermentation conditions (Table 1), and the protein digestibility activity by trypsin and pepsin.

MATERIALS AND METHODS

Fermentation conditions

Sample preparation and fermentation procedures were performed according to the method previously described by Mohd-Razali *et al*. (2019). Based on the previous study, three solid-state fermentation (SSF) conditions (Table 1) were used. Sampling activities were carried out every two day-interval, five days after fungal inoculation (DAI) until 13 DAI. Upon harvest, the total soluble protein and phytic acid contents were analyzed. Also, the protein digestibility rate (%) by trypsin and pepsin were determined on samples that encompassed the highest protein content, which was nine or eleven DAI (Table 2), respectively. Triplicates were applied for each condition.

Soluble protein and phytic acid contents

The soluble protein was extracted using the mild-acid extraction method as described by Pickardt *et al*. (2009) with slight modification. Three grams of samples were mild-acid extracted in 2.0 mL McIlvaine buffer (pH 4.0). The mixture was centrifuged (Eppendorf, USA) at 7000 × *g* for 30 min at 4°C. The total protein content in the supernatant was determined using the Bradford method (1976) at wavelength 595 nm based on the standard curve of the bovine serum albumin (BSA) in the range of 2.0 to 10.0 µg/mL.

The phytic acid content was determined according to Sadasivam and Manickam (1996). Five hundred mg of dried and finely pounded sample was extracted in 50 mL of 3% (w/v) trichloroacetic acid (TCA), with continuous shaking for 30 min. The mixture was then centrifuged at 3000 × *g* for 10 min. The supernatant (10 mL) was mixed with 4.0 mL of

Table 1. The solid-state fermentation conditions of the coconut residue samples

Conditions	Sample		
	A	B	C
Size of Inoculum (%)	10	20	30
Temperature of Incubation (0°C)	30	30	40
Salt Level (x times)	3	2	3

Table 2. The total soluble protein content in three selected solid-state fermentation conditions (Sample A, Sample B, and Sample C) at different days of fungal inoculation (DAI). (n =3; mean ± SE). A similar letter in the same column indicates no significant difference, $p<0.05$ by Tukey test)

Day after fungal inoculation (DAI)	Total soluble protein content in fermented coconut residue (mg/g dry wt. sample)		
	Sample A	Sample B	Sample C
5	23.25 ± 0.93^{c}	26.51 ± 2.32^{b}	35.81 ± 1.40^{c}
7	30.25 ± 2.32^{b}	30.70 ± 2.32^{a}	35.81 ± 1.86^{c}
9	39.41 ± 2.10^{a}	32.10 ± 0.93^{a}	37.67 ± 0.46^{c}
11	37.67 ± 1.40^{a}	36.94 ± 3.28^{a}	51.21 ± 0.67^{a}
13	36.27 ± 1.39^{a}	33.48 ± 3.25^{a}	45.58 ± 1.39^{b}

0.03% (w/v) $FeCl_3.6H_2O$ and heated in a water bath for 45 min followed by centrifugation at 3000 × *g* for 10 min. The precipitate was recovered and soaked in 20 mL of 3% (w/v) TCA, re-centrifuged at 3000 × *g* for 10 min, and rinsed with distilled water. Subsequently, the sample was dispersed in 3.0 mL of 1.5 M NaOH and distilled water to 30 mL final volume and heated for 30 min. The precipitated sample was attained by filtering using a Whatman filter paper (No. 2) and washed with 60 mL of hot distilled water. The precipitate was dissolved in 40 mL of hot 3.2 N HNO_3 and made up the volume to 100 mL with distilled water. 5 mL of aliquot was diluted with distilled water and 20 mL of 1.5 M KSCN to 100 mL. Subsequently, absorbance was measured using a spectrophotometer at 480 nm. The phytic acid contents were calculated based on a standard curve prepared from a stock solution of 433 mg Fe $(NO_3)_3$ in 100 ml dH_2O.

In-vitro protein digestibility by trypsin and pepsin

The *in-vitro* digestibility rate by trypsin was conducted according to the method of Yin *et al*. (2008) with modification. The soluble protein sample was diluted with 10 mm phosphate buffer (pH 8.0) to the final concentration of 2.00 mg/mL. One mg/L of trypsin was added to the protein solution, and the mixture was incubated in a water bath at 37°C. Samples were taken out at every 30 min interval until 120 min. The enzyme reaction was stopped by adding an equal volume of 20% (w/v) trichloroacetic acid (TCA). Precipitated protein was removed by centrifugation at 10,000 × *g* for 20 min.

Meanwhile, the *in-vitro* digestibility rate by pepsin was conducted according to the modified method by Bamdad *et al*. (2009) and Mirzakhani *et al*. (2018). One hundred mL of protein solution was adjusted to pH 2.0 using 1 N HCl and pepsin was added at 250:1 (v/v) substrate: enzyme ratio. The mixture was incubated in a water bath at 37°C and samples were taken at every 30 min interval until 120 min. The reaction was stopped by adding 1.0 M NaOH until pH 5.0 and boiled in a water bath for 10 min. Subsequently, the protein content was determined using the Bradford method (1976) as previously described. Three replicates were used for each condition.

Statistical analysis

All data obtained were analyzed using a one-way analysis of variance (ANOVA: SPSS version 16.0) followed with Tukey test for Post Hoc Multiple Comparisons.

RESULTS AND DISCUSSION

Soluble protein and phytic acid contents

Results in Table 2 show the soluble protein content from the selected fermentation conditions. The soluble protein content was dependent on the fermentation period and the fermentation conditions (Table 1). The highest soluble protein content for Sample A, B, and C was after 9, 7, and 11 DAI, respectively. Sample C contained the highest inoculum size (30%), however, it occupied the longest incubation period although it produced a significantly highest protein content ($p<0.05$) among the samples. The results suggested that the amylolytic enzymes such as the α-amylase, glucoamylase, and α-glucosidase might be activated at higher incubation temperature, which ranges from 37 to 40°C (Futagami *et al.*, 2015).

Results in Figure 1 clearly show that the fermentation period significantly contributed to

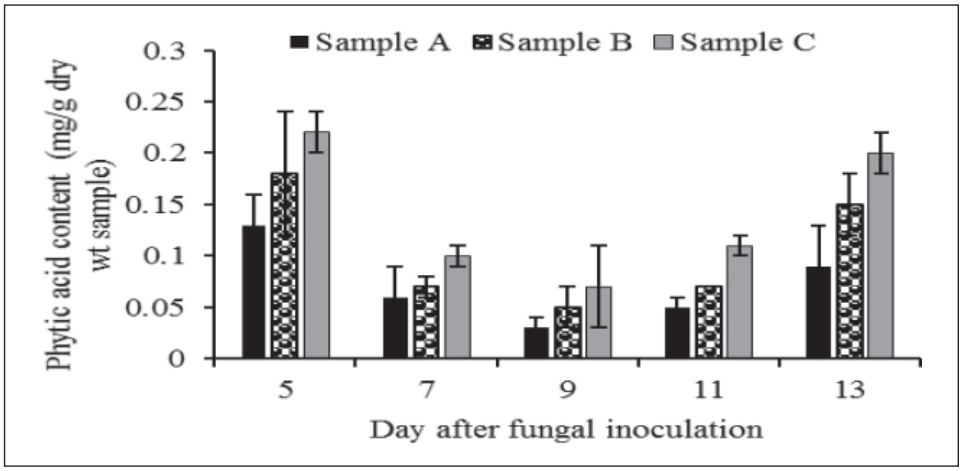

Fig. 1. The phytic acid content in coconut residues from three selected solid-state fermentations at days of fungal inoculation (DAI). Bar indicates the SE, significant difference, $p<0.05$ by Tukey test (n =3).

the phytic acid content. For all samples, the lowest phytic acid content was determined at 9 DAI, which was in a very low range, from 0.03 to 0.07 mg/g dry wt. of the sample. However, there is no significant difference (p>0.05) among the treatments at those incubation periods (Figure 1). After that, the phytic acid increased until 13 DAI. The finding showed that the phytic acid and soluble protein ratio is antagonistic. The results suggested that the fermentation process decreased the amount of phytic acid content in the substrate as the fungal growth and development absorbed the nutrients supplied in the medium (Ramachandran *et al*., 2004). It is considerably proposed that the optimum time for fermentation of coconut residue was at 9 DAI. The divergent connections of both are hypothetically presumed due to microbial phytase produced during fermentation in conjunction with the protein production. Phytic acid might also form a complex with protein (Kies *et al*., 2006), and is not limited to the cations such as Ca, Mg, K, Fe, or Zn. A previous study showed that 2 to 3% of soy protein formed were strongly bonded to phytic acid (Rizzo & Baroni, 2018). The formation of the phytate-protein complex is dependent on the type of protein, pH, the presence of Ca and Mg, protein solubility rate, proteolytic enzymes, and protein content (Kies *et al*., 2006). The complex formation may lead to a decreased protein digestibility in farm animals (Cowieson *et al*., 2006). Phytic acid chelates with minerals in the small intestine at alkaline conditions and forms a poorly soluble complex of phytate (Kemme *et al*., 1999; Gilani *et al.*, 2005).

In-vitro protein digestibility by trypsin and Pepsin

The main concern in microbial-based derived-protein is the digestibility of stomach proteases, trypsin, and pepsin. Results of the current study shows that protein digestibility by trypsin on all samples were in the recommended range of digestible protein as desired for aquaculture feed. Generally, fermented coconut residues derived-protein digestibility by both trypsin and pepsin were high after 90 min of incubation for all samples (Figures 2 and 3). Protein digestibility by trypsin for samples A, B, and C were at 43.22 ± 2.46, 37.24 ± 5.62, and 36.54 ± 4.22% after 120 min of digestion, respectively. These rates of protein digestibly were high and fell within the recommended range for fish feed formulation, which is 20 to 45% (El-Sayed *et al.*, 1999; Abdelnour *et al.*, 2018).

On the other hand, the highest *in-vitro* protein digestion by pepsin was discovered after 120 min of incubation (Figure 3) and it was lower compared to trypsin. The highest protein digestibility by pepsin in samples A, B, and C were 19.58 ± 4.17, 23.53 ± 2.92, and 15.33 ± 5.5%, respectively. The least protein digestibility might be due to the peptide bond formed among the non-cleavable amino acid by pepsin such as valine and alanine (Mirzakhani *et al*., 2018). Additionally, the microbial phytase was reported to decrease protein precipitation but increases protein digestibility. The characters of the effective digestibility rate by trypsin and the presence of pepsin-non-cleavable amino acid suggested feed compatibility for animals with a highly efficient digestion system. Nonetheless, the herbivorous fish such as tilapia or catfish with smaller stomach and long intestinal tract as compared to carnivorous fish could permit nutrient efficiency absorption (Farizaldi *et al.*, 2017; Mirzakhani *et al*., 2018). Also, the fermented coconut residues-derived protein might be suitable

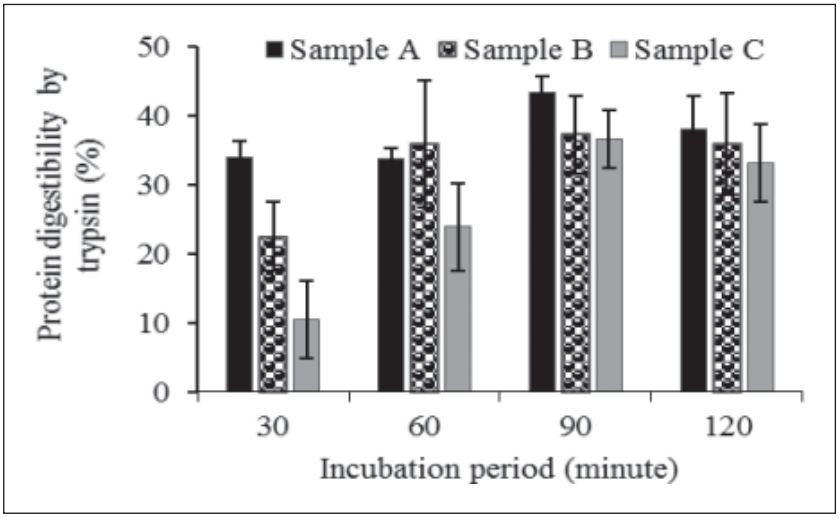

Fig. 2. The protein digestibility by trypsin in coconut residues from three selected solid-state fermentation. Bar indicates the SE, significant difference, $p < 0.05$ by Tukey test (n =3).

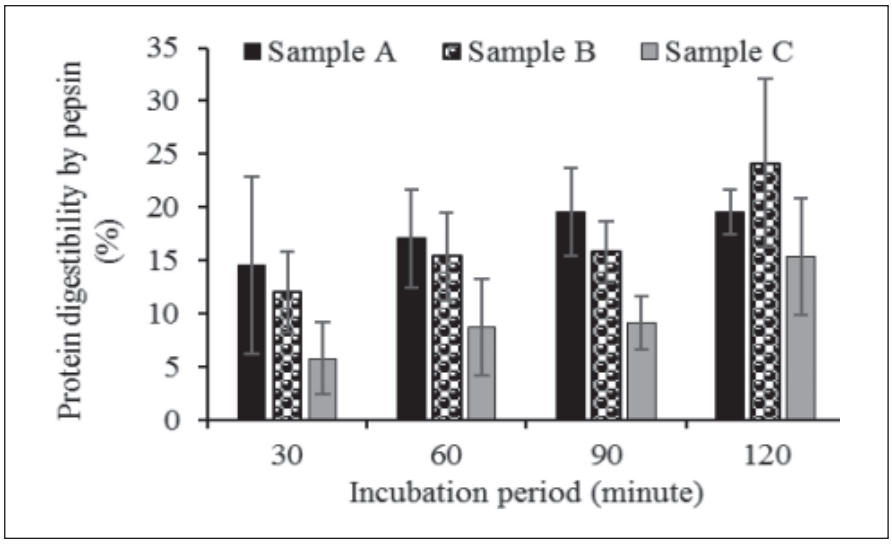

Fig. 3. The protein digestibility by pepsin in coconut residues from three selected solid-state fermentation. Bar indicates the SE, significant difference, $p<0.05$ by Tukey test (n =3).

for the larvae of Asian sea bass (*Lates calcarifer)* with the absence of stomach and probably usage of the stomach alkaline protease (pH 8.0), which not related with pepsin (Ronnestad *et al*., 2003). Therefore, the ability of *A. awamori* to support the improvement of protein digestibility quality from coconut residue through solid-state fermentation might be the key to a successful increment in processed protein.

CONCLUSION

The solid-state fermentation of coconut residues by *A. awamori* had increased the soluble protein content with lower phytic acid, the anti-nutritional substance after nine DAI. The amount of soluble protein and phytic acid, together with protein digestibility were influenced by the period of fermentation. The fermented coconut residues-derived protein exhibits high protein digestibility by the intestine enzyme trypsin than the stomach enzyme pepsin. The findings suggested that the derived-protein could be proficient as a feed for herbivorous fishes. This opens to a new insight in bioconversion of agro-waste into wealth, where the fermented coconut residue is a renewable sparing protein and/or energy in feed formulation.

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