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Effects of fishmeal replacement by soybean meals treated with lignocellulolytic enzymes from spent mushroom substrate on growth performance, biochemical parameters and hepatopancreas histology of white shrimp (*Litopenaeus vannamei*)

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Abstract After 8 weeks of feeding white shrimp with lignocellulolytic enzynes-treated soybean meal (ESBM) replacing fishmeal diet, no significant difference of the growth performance was observed among the shrimp fed with 0% ESBM, 25% ESBM, 50% ESBM and the control (commercial diet). In contrast, shrimp fed with a diet with non-treated soybean meal (SBM) showed significantly lower growth performance ($P<0.05$). Correspondingly, the serum biochemical parameters, AST, SOD and MDA, showed no significant difference among the 0%-50% ESBM. Moreover, the histological examination of hepatopancreas revealed that the treatments of 0% ESBM, 25% ESBM, and 50% ESBM provided good effects on health condition. Altogether, 50% ESBM could be the highest level of the treated SBM to replace fishmeal in white shrimp diet.

Keywords: Soybean meal, Fishmeal replacement, Lignocellulolytic enzyme, Growth performance, White shrimp (*Litopenaeus vannamei*)

Introduction

White shrimp, *Litopenaeus vannamei*, is the most widely cultured shrimp species because of a robust growth in market demand, production growth, and profitability if properly managed (Karim *et al*., 2014; Rego *et al*., 2017). As shrimp production has grown rapidly for decades, the requirement of fishmeal, an important and widely used as the most suitable protein source in shrimp diet,

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increases coressondingly. This may be due to fishmeal contains proper nutrient characteristics and quality. It has been considered as a good source of essential amino acid, fatty acid, vitamins, minerals as well as its attractability and palatability (Dantas Jr *et al*., 2016; Davis and Arnold, 2020). However, due to the high price of fishmeal and the reduction of fisheries resources, its application in aquafeed production is limited (Yan *et al*., 2014). There has been proposed that finding sustainable protein sources for feed is crucial to not only reduce dependence on wild fish stocks but also minimize the environmental impact of aquaculture. Therefore, many researchers have focused on discovery of alternative protein sources, at the proper inclusion level, to replace fishmeal in shrimp feed (Xie *et al*., 2016; Zhao *et al*., 2016; Shao *et al*., 2017; 2019; Novriadi *et al*., 2022).

Soybean meal (SBM) has been reported as a plant protein that can be used for fishmeal substitution because of its availability and lower cost. Additionally, its nutritional characteristics of high protein content and amino acid profile make SBM more interesting (Yun *et al*., 2017). Nevertheless, one of the impportant issues with the SBM usage is an absence of essential amino acids, for example, lysine and methionine, which limits its use in shrimp feed (Yue *et al*., 2012). Furthermore, several anti-nutritional factors such as protease inhibitors, phytic acid, tannins, saponins lectins, alkaloids, and non-starch polysaccharides have been found in SBM. These substances are able to interfere with nutrient and might cause inflammation in shrimp digestive tract (Gatlin *et al*., 2007; Krogdahl *et al*., 2010; Yun *et al*., 2017). Previous studies have demonstrated that high level of SBM in the diet could reduce growth performance, antioxidative capacity, and digestibility, and cause induced damage of digestive tract of aquatic animals including shrimp (Ostaszewska *et al*., 2005; Lin and Mui, 2017). Treatment by fermentation with microorganisms, for example, *Bacillus subtilis*, *Lactobacillus* spp. and *Saccharomyces cerevisiae*, and certain types of degeading enzymes have been reported to improve quality of SBM by reducing negative effects and increase its utilization for white shrimp (Zhuo *et al*., 2014; Van Nguyen *et al*., 2018; Shao *et al*., 2019).

Since carbohydrates in SBM consist mainly of non-starch polysachharides (NSP) which cannot be digested by shrimp, one of the treatment procedures to improve the SBM quality is fermentation with NSP enzyme. In this present work, a cocktail mix of lignocellulolytic enzymes obtained from the the mushroom spent substrate of split gill mushroom and oyster mushroom was used to treat SBM producing enzyme-treated SBM or ESBM. The effects of fishmeal replacement by ESBM at different levels on the growth performance and hepatopancreas of white shrimp were investigated in comparison with the commercial diet and SBM diet.

Materials and methods

Preparation of ESBM

ESBM was prepread by mixing the thoroughly ground soybean meal with the mixed lignocellulolytic enzymes extracted from the spent mushroom substrates of split gill mushroom and oyster mushroom (after harvest). After incubation at 50ºC for 24 h, the treated ESBM was oven-dried prior to be kept at -20ºC until used.

Experimental diets

There were 6 diets used in this experiment, 1 was commercial diet and 5 diets prepared in the laboratory. Every diet formula was fixed to contain approximately 38% crude protein and 7.5% crude lipid. Proximate analysis and formulation of the trail diets, except that of the commercial diet, is presented in Table 1. Five trial diets: (1) SBM, (2) 0%ESBM or 100% fishmeal, (3) 25%ESBM, (4) 50%ESBM, and (5) 100%ESBM which refered to fishmeal substitution by ESBM at 25%, 50% and 100%, respectively, were prepared. The diets were manufactured followed the method of Chen *et al*. (2018). All experimental diets were oven-dried and dehumidified in an air-conditioned room (24ºC) until the moisture was around 8-10% and then kept at -20ºC until used. The commercial diet containing the similar nutritional composition mentioned before for the experimental diets was used as the control.

Experimental procedure and shrimp rearing

Juveniles white shrimp (mean weight 1±0.2 g) were bought from a commercial shrimp farm in Nakhon Si Thammarat Province. Shrimps were acclimatized in a plastic tank containing 2,000 L of sea water (10 ppt) under controlled laboratory condition. Shrimps were fed apparent satiation with the commercial diaet 4 times a day. After a week, shrimps with average body weight of 2±0.3 g were randomly selected and separated into 24 of 200 L plastic tank (20 shrimps/tank) for 6 treatments 4 replicates. Shrimp were fed with the respective experimental diets for 8 weeks meanwhile feed consumption was recorded daily. The rearing condition was controlled as follow; water temperature ranged from 24 to 28°C. pH 7.6-7.8, and salinity 10 ppt, throughout the experimental period. Total ammonia nitrogen (TAN) and dissolved oxygen (DO) was less than 0.05 mg/L and above 7.0 mg/L, respectively.

Table 1. Formula and proximate composition analysis of the experimental diets (% dry matter)

Ingredient (%)	Diet (Fish meal protein replacement (%))				
	Diet 1 (SBM)	**Diet 2 (0%ESBM)**	**Diet 3 (25%ESBM)**	**Diet 4 (50%ESBM)**	**Diet 5 (100%ESBM)**
Fish meal	0.00	30.00	22.50	15.00	0.00
Enz- treated soybean meal (ESBM)	0.00	0.00	10.27	19.19	36.90
Soybean meal (SBM)	36.395	9.03	5.53	3.56	0.00
Cellulose	0.00	9.03	5.53	3.56	0.00
Fish oil	4.50	3.00	3.58	3.98	4.53
Mineral mixtur [a]	1.00	1.00	1.00	1.00	1.00
Vitamin mixture[b]	1.00	1.00	1.00	1.00	1.00
Ascorbic acid	0.015	0.015	0.015	0.015	0.015
Monocalcium phosphate	1.00	1.00	1.00	1.00	1.00
Lysine	0.40	0.00	0.10	0.20	0.40
Methionine	0.20	0.00	0.05	0.10	0.20
Others (e.g. poultry meal)	54.95	54.95	54.95	54.95	54.95
Total		100.00	100.00	100.00	100.00
Proximate analysis (%)					
Dry matter	90.23	89.91	89.77	88.89	90.02
Crude protein	38.35	38.52	38.24	38.47	38.73
Crude lipid	7.65	7.65	7.89	7.52	7.79
Crude ash	10.01	10.10	9.84	9.93	9.68

[a] Mineral mixture (mg/kg of diet): $FeSO_4 \cdot H_2O$, 30.41; $CuSO_4 \cdot H_2O$, 41.91; $ZnSO_4 \cdot H_2O$, 274.34; $MgSO_4 \cdot H_2O$, 284.47; $Ca(IO_3)_2$, 6.14; Na_2SeO_3, 0.44; $CoSO_4$, 2.89.

[b] Vitamin mixture (per kg of diet): vitamin A, 12000 IU; riboflavin, 40 mg; cyanocobalamin, 0.02 mg; thiamin, 50 mg; menadione, 40 mg; vitamin C, 250 mg; folic acid, 10 mg; calcium pantothenate, 100 mg; nicotinic acid, 120 mg; biotin, 1 mg; vitamin D3, 2000 IU; a-tocopherol, 120 mg; pyridoxine HCl, 60 mg.

Sample collection

After experimental feeding for 8 weeks, all shrimp were straved for 24 h and bulk weighed, then shrimp in each tank were recorded for growth performance analysis. Hemolymph was individually collected from 7 shrimp

from each tank, stored at room temperature for 1 h then at 4°C for 3 h followed by centrifugation at 4°C, 3,000 rpm for 10 min (Supamattaya *et al*., 2005). The clear supernatant was pooled and kept at -80°C until determination of serum biochemical indicators. Hepatopancreas sample was collected from 3 shrimp/tank and stored in Davidson's AFA fixative (DAFA). After 24 h, the samples were stored in 70% ethanol for tissue section preparation.

Growth performance and biochemical indicators

The growth performance indicators were calculated as follow:

Weight gain (WG, g) = final weight – initial weight.

Average daily weight gain (ADG, g/d) = (final weight – initial weight) / culture time.

Feed conversion ratio (FCR) = total feed intake / total weight gain.

Specific growth rate (SGR, %/d) = 100 x (*ln*final weight – *ln*initial weight) / culture time.

Survival rate (SR, %) = ultimate number / initial number x 100.

The analysis of serum biochemical parameters including activity of superoxide dismutase (SOD), catalase, aspartate aminotransferase (AST), and malondialdehyde (MDA) referring to lipid peroxidation were performed. These parameters were determined by a microplate reader using using commercial kits (Sigma-Aldrich).

Hepatopancreas histolology

The hepatopancreas samples stored in 70% ethanol were dehydrated in gradient concentrations of ethanol, paraffin embedded and then cut at a thickness of 5 μm by using a microtome. The sections were stained with haematoxylin and eosin (H&E) following the protocol of Goldner (1938) and observed under a light microscope (Nikon).

Statistical analysis

Data are presented as mean ± SD. One-way analysis of variance (ANOVA) using SPSS Statistics software version 16.0 (SPSS Inc.) was used to verify the statistical differences of each data set. Significant differences were evaluated by Duncan’s Multiple Range Test (DMRT), and stated at $P<0.05$.

Results

Growth performance

The growth performance is shown in Table 2. Shrimp fed with the diet containing non-treated soybean meal, SBM, showed lowest growth performance indices. Comparison with the control group, shrimp fed with commercial diet, the results revealed that WG was not significant in the group fed with the 0%ESBM or fishmeal diet (P>0.05) while it decreased in other diets that replace fishmeal with 25%, 50% and 100% of ESBM. Similar results compared among treatments were observed for SGR, ADG, FCR and survival rate. No significance was detected in control, 0%ESBM, 25%ESBM and 50%ESBM while it was statistically decreased in 100%ESBM.

Table 2. Effects of enzyme-treated soybean meal (ESBM) substituting fishmeal on growth performance of *L. vannamei*

Parameter	**T1 (Control)**	**T2 (SBM)**	**T3 (0%ESBM)**	**T4 (25%ESBM)**	**T5 (50%ESBM)**	**T6 (100%ESBM)**
IW (g)	2.14±0.10	2.23±0.04	2.22±0.02	2.23±0.02	2.18±0.10	2.24±0.10
FW (g)	15.29±0.51^{c}	10.94±0.57^{a}	15.13±0.45^{c}	14.99±0.51^{c}	14.79±0.65^{c}	12.29±0.51^{b}
WG (g)	13.36±0.43^{d}	8.31±0.54^{a}	13.24±0.44^{d}	12.81±0.54^{c}	12.66±0.63^{c}	10.36±0.43^{b}
SGR (%/d)	3.47±0.03^{c}	2.84±0.06^{a}	3.43±0.05^{c}	3.40±0.08^{c}	3.42±0.09^{c}	3.04±0.03^{b}
ADG (g/d)	0.27±0.01^{c}	0.20±0.01^{a}	0.27±0.03^{c}	0.27±0.04^{c}	0.25±0.01^{c}	0.22±0.01^{b}
FCR	1.35±0.02^{a}	1.63±0.06^{a}	1.36±0.04^{ab}	1.39±0.06^{b}	1.37±0.02^{ab}	1.48±0.02^{c}
SR (%)	87.67±1.46^{b}	80.50±2.89^{a}	85.00±4.08^{b}	87.50±2.89^{b}	86.97±1.53^{b}	83.67±1.53^{ab}

IW, initial weight; FW, final weight; WG, weight gain; SGR, specific growth rate; ADG, average daily growth; FCR, feed conversion ratio; SR, survival rate.
Results are presented as the mean ± SD (n = 4). Different superscript letters in the same row indicate statistically significant differences (P<0.05).

Serum biochemical parameters

The activities of SOD and catalase was comparable compared among treatments (Table 3). Fishmeal replacement with 25%ESBM and 50%ESBM redeuced these enzyme activities while 100%ESBM and SBM showed the lowest activity ($P<0.05$). In caontrast, high level of ESBM substitution increased the AST activity and MDA content ($P<0.05$).

Table 3. Effects of enzyme-treated soybean meal (ESBM) substituting fishmeal on serum biochemical indices of *L. vannamei*

Parameter	T1 (Control)	T2 (SBM)	T3 (0%ESBM)	T4 (25%ESBM)	T5 (50%ESBM)	T6 (100%ESBM)
SOD (U/ml)	24.34±2.13^{c}	21.26±2.56^{a}	23.12±1.78^{bc}	22.67±1.89^{b}	22.34±1.79^{b}	21.34±0.89^{a}
Catalase (U/ml)	1.06±0.03^{cd}	0.65±0.11^{a}	0.89±0.07^{bc}	0.97±0.08^{c}	0.84±0.11^{bc}	0.73±0.08^{ab}
AST (U/ml)	7.24±0.36^{a}	9.75±0.52^{c}	7.45±0.35^{a}	7.57±1.04^{ab}	8.05±0.24^{b}	9.13±0.47^{c}
MDA (mmol/mg)	9.53±0.71^{a}	12.46±0.67^{c}	10.05±0.63^{a}	11.15±0.52^{b}	10.75±0.73^{ab}	10.98±1.21^{b}

SOD, superoxide dismutase; AST, aspartate aminotransferase, MDA, malondialdehyde. Results are presented as the mean ± SD (n = 4). Different letters in the same row indicate statistically significant differences ($P<0.05$).

Hepatopancreas histology

The sections of hepatopancreatic tissue of the shrimp fed with the trial diets are shown in Figure 1. Well-organized tubular structure and intact cells, B-cell, R-cell, and F-cell, as normally seen in *L. vannamei* was observed in the control showed the (Figure 1A). The similar observed structures were also present with regular lumen shape in the group fed with 0%ESBM, 25%ESBM and 50%ESBM shown in Figure 1C, 1D and 1E, respectively. These results suggested that fishmeal replacement with 25% and 50%ESBM gave no toxicity to the shrimps. Some of the lumen shape was found distorted and irregular in the shrimp samples fed with SBM (Figure 1B) and 100%ESBM (Figure 1F). Delamination, atrophy, and cell sloughing in hepatopancreas, and decrease of the B-cell number and size was found particularly in SBM-fed *L. vannamei* while some of these were observed in 100%ESBM.

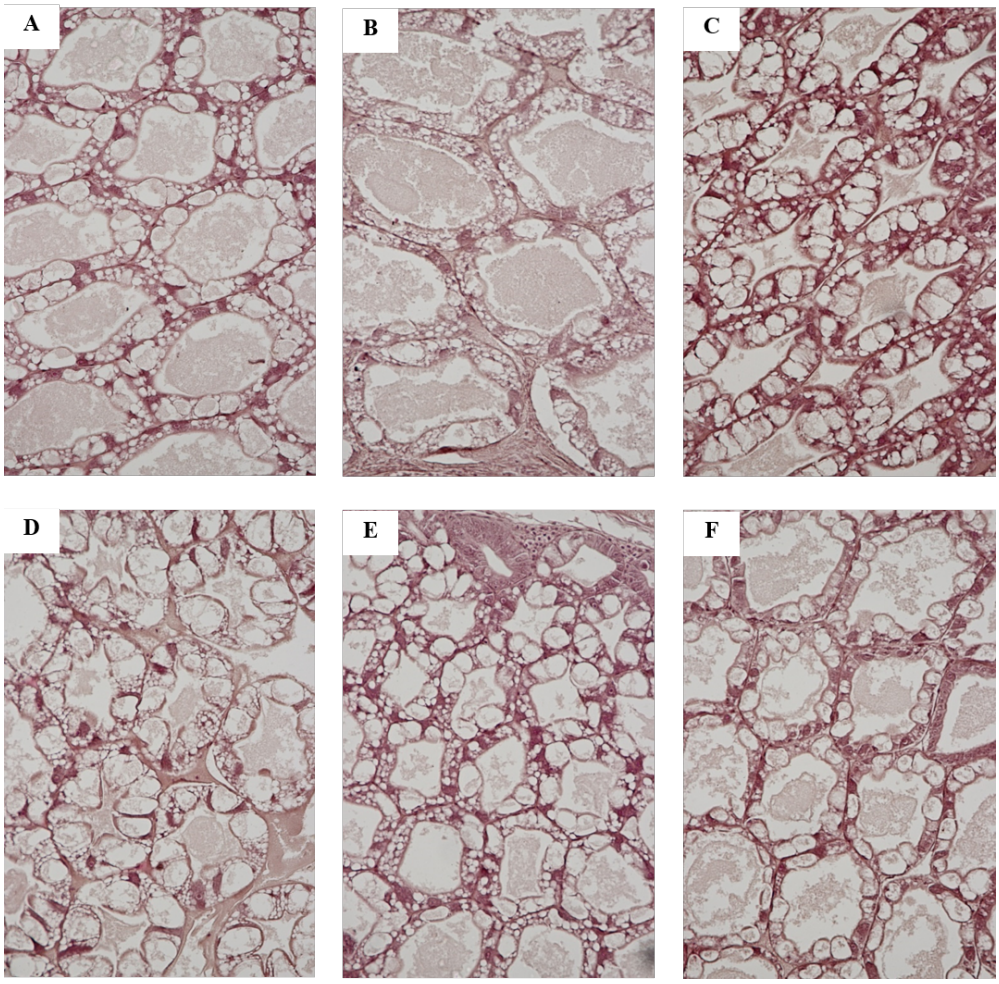

Figure 1. Hepatopancreas histology of *L. vannamei* (Magnification: 400X). Shrimps were fed with the trial diets with different percentages of fishmeal replacement with lignocellulolytic enzyme-treated soybean meal (ESBM). **(A)** Control or commercial diet, **(B)** SBM, **(C)** 0%ESBM, **(D)** 25%ESBM, **(E)** 50%ESBM, and **(F)** 100%ESBM. Sections were stained with haematoxylin and eosin (H&E)

Discussion

Because of the rapid expansion of shrimp culture and requirement of fishmeal as the shrimp feed ingredient, research on finding substitution of fishmeal has become an important issue. Concern about the NSP content (mainly cellulose and hemicelluloase) and anti-nutritional factors become a serious problem since these constituents have been reported to exhibit negative effects

on shrimp health. Shrimp lacks digestive enzymes to degrade NSP, therefore, high dietary NSP level may negatively affect digestive processes through increasing viscosity and transit velocity rate of digesta, altering intestinal morphology, and gut microbiota resulting in intestinal dysfunction (Sinha *et al*., 2011). Lignocellulolytic enzymes consisting of various glycoside hydrolase (mainly cellulases and hemicellulase) (Gao *et al*., 2023) become an interesting tool for degrading NSP in SBM. Diverse organisms capable of producing these enzymes include fungi, bacteria, actinobacteria and yeast. White rot fungi such as split gill mushroom (*Schizophyllum commune*) and oyster mushroom (*Pleurotus ostreatus*) have been considered as good microbes exhibiting high ability to degrade lignocellulose. Our previous work has demonstrated that the spent mushroom substrate of these fingi contained 2 major groups of enzymes. First group is ligninolytic enzymes including laccase, peroxidase, lignin peroxidase (LiP), and manganese peroxidase (MnP). Another group is cellulolytic enzymes, endoglucanase (EG), exoglucanase or cellobiohydrolase (CBH), and β-glucosidase (BGL). Moreover, treatment with these enzymes shows increasing of protein digestibility and reduction of trypsin inhibitor in the treated SBM.

The feasibility of using the ESBM was evaluated, the SBM treated with the lignocellulolytic enzymes from spent mushroom substrates, to replace SBM in *L. vannamei* diet. Comparison with the control group fed with commercial diet, 25%ESBM and 50%ESBM exhibited lower FW, WG and SGR but not SR. However, the growth performance indicators did not differ compared to that fed with 0%ESBM or 100% fishmeal ($P>0.05$). The shrimp growth significantly declined to be the same result of using SBM when the ESBM level was too high (100% fishmeal replacement). In the growth aspect, our finding was comparable with other research works on fishmeal substitution by fermented soybean meal. Several researchers have demonstrated that the fermented soybean meal can be partially replaced fishmeal at certain level (around 20%-40%) in shrimp diet with unaffected results (Zhuo *et al*., 2014; Van Nguyen *et al*., 2018; Shao *et al*., 2019; Bae *et al*., 2020; Lin and Chen, 2022).

Biological markers such as serum AST and MDA have offen been used as indicators for tissue damage and lipid peroxidation reflecting health status of living organisms (Zhou *et al*., 2015). Increase of MDA, a product of lipid peroxidation, indicates an excessive level of oxygen free radicals which refers to the oxidative damage level of cells (Zhang *et al*., 2022). Oxidative active enzymes or antioxidant-related enzymes, for example, SOD, peroxidase (POD) and catalase play an important role in shrimp immune responses. SOD catalyzes the conversion of superoxide anion into oxygen and hydrogen peroxide, which increases the defense potential of phagocytes (Yang *et al*., 2010). POD and

catalase can effectively eliminate reactive oxygen species and other harmful substances produced in metabolisms, hence reducing cell damage (Zhang *et al*., 2022). The SOD activity in the shrimps fed with 25%ESBM and 50%ESBM were statistically lower than the control ($P<0.05$) while catalase was not significant ($P>0.05$). The indicators regarding tissue damage were not affected by substitution of ESBM at the levels of 0%-50% compared to those detected in the control group. Our results suggested that ESBM could be replaced fishmeal at 25%-50%.

Analysis of hepatopancreas histology has been accounted as one of the considerable indices for reflecting shrimp's health status (Wu *et al*., 2008; Sun *et al*., 2015). In the present study, the structures of hepatopancreas in shrimp fed with 0%ESBM, 25%ESBM and 50%ESBM diets were normal and intact similar like that present in the control group, suggesting replacement fishmeal with ESBM at the mentioned levels did not negatively affect or cause damage to the hepatopancreas.

In summary, this present work provided the evidence suggesting that the ESBM could be a good candidate for protein source replacing fishmeal in shrimp diet. Our prepared lignocellulolytic enzyme cocktail from spent mushroom substrates was able to improve the quality of SBM to make its fermented form suitable for fishmeal substitution. Altogether, 50% ESBM could be the highest level of the treated SBM to replace fishmeal in the practical diet without adverse effects on the growth, health status and hepatopancrase histology of white shrimp *L. vannamei*.

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