WALAILAK JOURNAL
http://wjst.wu.ac.th Applied Sciences

Effect of *Aspergillus niger* Fermented Soybean Meal Supplementation in Formulated Diets on Growth Performances in Juvenile Asian Seabass (*Lates culcarifer* Bloch)

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Received: 11 April 2018, Revised: 12 February 2019, Accepted: 13 March 2019

Abstract

The utilization of indigenous mold *Aspergillus niger* from aquaculture areas to improve the quality of soybean meal (SM) by fermentation and replacement in Asian seabass diet was studied on growth performances and feed utilization in juvenile Asian seabass, *Lates calcalifer*. Sinking diets with 40 % of protein were formulated. The main sources of protein in the diets were from fish meal (FM). Low substitution levels of 4 % of protein from SM, 10 % of total protein, were used in diets D1 - D4 with replacement of fermented soybean meal (FSM) at 0, 25, 75, and 100 %, respectively, and high substitution levels of 8 % of protein from SM, 20 %of total protein, were used in diets D5 - D8 with replacement of FSM at 0, 25, 75, and 100 %, respectively. Initial weight of juvenile Asian seabass was 12.83 - 12.90 g. 10 fish were stocked in 100 L glass tanks with triplicate groups. Each diet was fed to apparent satiation twice daily for 10 weeks. The results presented that the final body weight (BW), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and feed capital showed significant difference ($P < 0.05$) while final total length (TL), survival rate (SR), carcass percentage, and hepatosomatic index (HSI) did not show significant difference ($P > 0.05$). The 4 % of protein from SM was able to be replaced by 25 - 100 % of FSM with increased growth performances and feed utilization, while feed cost (Thai baht kg^{-1} of fish) was reduced, respectively. The *A. niger* FSM is alternative protein source for growth, feed utilization improvement, and feed cost reduction of Asian seabass farmers.

Keyword: Fermented soybean meal, Indigenous *Aspergillus niger*, Juvenile Asian seabass

Introduction

Asian seabass, *Lates calcalifer* Bloch, 1970, is a brackish water fish species that is widely distributed in the Persian Gulf, throughout India, Southeast Asia, Northern Australia, Papua New Guinea, and the western Pacific. It is a significant culturally and economically important species in tropical Australasia, Asian countries bordering the Indian Ocean [1], and Thailand. The Asian seabass production of Thailand was 16,501 tons with the value of 2,029.96 million baths in 2014 [2]. The main cost of seabass production is feed, 50 - 70 % of total production cost. The major source of protein in aquafeed is fish meal. The variability of landing of fish used to make fish meal has led to a decrease in fish meal production, while the price of the fish meal has increased due to high demand for aquafeed industries, causing the price of feed becoming more expensive [3]. Fish meal with plant protein ingredients is an alternative to reduce fish meal used and decrease feed price. Soybean meal is an ingredient widely used in aquafeed formulations. However, soybean meal contains a variety of antinutritional substances, protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, and allergens [4]. There is a decrease of nutritional utilization of feed and growth performance in animals [5]. Therefore, usage of soybean meal is limited in aquafeeds, as Lee *et al.* replaced 20 - 40 percent of fish meal protein by soybean meal in juvenile rockfish, *Sebastes schlegeli,* resulting in decrease in feed intake, feed efficiency (FE), protein efficiency ratio (PER), and growth rate [6]. Romarheim *et al*., Heikkinen *et al*., and Merrifield *et al*. reported that usage of soybean meal in rainbow trout, *Oncorhynchus mykiss*, resulted in reduced growth and morphological abnormality of the distal intestine, as well as changes in a microbial community in the intestine [7-9].

Antinutritional substances in feed material can be reduced by various methods: heating, a combination of heating and solvents [10], and infrared radiation [11]. Heating by autoclave for 15 - 30 min reduced trypsin inhibitors in soybean meal, leading to them being under critical levels [12]. However, heating can destroy heat-labile factors; protease inhibitors, phytates, lectins, and antivitamins, whereas heat stable factors; saponins, non-starch polysaccharides, antigenic proteins, estrogens, and some phenolic compounds remain [5]. Francis *et al.* mentioned that, although heating can destroy heat-labile antinutritional factors, it also affects the nutritional quality of feed materials, such as a decrease in protein digestibility due to excessive heat denaturation and loss of availability of lysine [4].

Fermentation is a method can reduce antinutritional substances in soybean meal [4]. The bacteria consume sugar from the plant by cellulose and carbohydrate digestion for their energy and produce volatile fatty acids (VFAs), such as acetic acid, lactic acid, propionic acid, [13] etc. Lactic acid affected the reduction of antinutritional substances [14]. The study of Hong *et al.* found that soybean meal fermentation by mold *Aspergillus oryzea* destroyed trypsin inhibitors and increased the number of small-size peptides (< 20 kDa) [15]. *A. niger* is a kind of mold that has a similar attribute to *A. oryzae*. *A. niger* is a black-spored filamentous fungus. It can be found in soil, compost, and decaying plant material. It is able to grow in a wide temperature range of 6 - 47 °C, with optimum growth at 35 - 37 °C [16]. The property of substance production of *A. niger* has been reported, such as citric acid, pectinase, amyloglucosidase [16], protease [17,18], cellulose and hemicellulases [19], lipase [20], phytase [21], tannase [22], etc. Moreover, *A. niger* is accepted as a safe organism for production of extracellular enzymes and citric acid for food industries and is considered Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration. It has never been identified as being the primary cause of any disease in human. [16].

An indigenous microorganism in the aquaculture area in the Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang campus, Thailand, was surveyed in order to its utilization for natural aquaculture, following the natural farming principles [23]. The theory of natural farming is producing high yields with minimal inputs, and utilizes local natural resources, including indigenous microorganisms [24]. The survey found several types of bacteria and mold, included *A. niger* [23]. The properties of *A. niger* were previously mentioned; soybean meal fermented by the indigenous *A. niger* in the formulated diet was estimated, in order to improve the growth and feed utilization of Asian seabass.

Materials and methods

Fermentation of soybean meal

Aspergillus niger was isolated from the aquaculture area in the Faculty of Science and Fisheries Technology, Trang campus. Fermentation of soybean meal (SM) was modified from the Yamamoto *et al.* [25] and Kim *et al.* [26] methods. *A. niger* hyphae were grown on PDA and used for *A. niger* starter production. The SMB was rehydrated with 30 % (w w^{-1}) of water for 30 min, put in a plastic bag, and sterilized for 15 min. After cooling, *A. niger* hyphae was inoculated into SM and incubated at room temperature for about 10 - 15 days, until the hyphae grew to the bottom of the bag. Fermented soybean meal (FSM) was produced, plain SM was rehydrated to be the same as the starter produce method, 10 % (w w^{-1}) of starter was added, and it was mixed well and incubated at room temperature for 48 h. The FSM was stocked in -20 °C for proximate analysis [27] and diet production.

Diet production

The isonitrogenous (40 %) and isolipidic (10 %) sinking diets were formulated (**Table 1**). Fish meal (FM) was used as the main protein source. 4 % of protein from SM (10 % of total protein in diet) was used in diets D1 - D4 with replacement of FSM at 0, 25, 75, and 100 %, respectively, and 8 % of protein from SM (20 % of total protein in diet) was used in diets D5 - D8 with replacement of FSM at 0, 25, 75, and 100 %, respectively. The other ingredients of the diets are shown in **Table 1**. The diets were stocked in -20 °C for proximate analysis [27] and the experiment.

Fish husbandry

Juvenile Asian seabass were stocked in a 3 m^3 tank with 15 ppt of water and trained with the fed control diet (D1) twice daily to apparent satiation for 2 weeks. After acclimatization, the fish were starved for 24 h [28] and anesthetized with 50 ppm clove oil. The body weight (BW) and the total length (TL) were measured individually. 10 uniform-sized fish (approximately 12 g body weight) were stocked in each of 30 glass tanks with 100 L of water volume and aeration. The fish were acclimatized to the experimental condition and fed control diet for a week. Each diet was fed to apparent satiation twice daily to 3 replicate tanks for 10 weeks [28].

Data collections

The measurement of final BW, TL, and survival rate (SR) were done at 10^{th} week individually. The amount of feed through the experiment was recorded for feed intake (FI), feed convention ratio (FCR), and protein efficiency ratio (PER) calculations. The edible portion and liver weights were determined for carcass percentage and hepatosomatic index (HSI) calculations.

Table 1 Composition (%), proximate analysis, and cost of the experimental Asian Seabass diets.

Ingredients (%)	**Experimental diets**							
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**
FM (64.73 % CP)	55.62	55.62	55.62	55.62	49.44	49.44	49.44	49.44
SM (41.40 % CP)	9.66	7.25	2.41	0	19.33	14.50	4.83	0
FSM (37.31 % CP)	0	2.68	8.04	10.72	0	5.36	16.08	21.44
Rice Bran	0	9.90	11.70	12.65	14.30	16.20	19.80	21.67
Cooked Rice Meal	17.12	15.90	13.46	12.19	9.93	7.40	2.51	0
Palm Oil	1.60	1.65	1.77	1.82	0	0.10	0.34	0.45
Vitamin Mix[a]	1	1	1	1	1	1	1	1
Mineral Mix[b]	1	1	1	1	1	1	1	1
CMC	5	5	5	5	5	5	5	5
Proximate composition (% dry matter)								
Protein	41.60	41.31	41.02	41.48	41.09	41.16	41.07	41.01
Lipid	10.73	10.35	10.29	10.42	10.40	10.39	10.35	10.40
Ash	18.26	18.21	18.22	18.30	18.00	18.21	18.09	18.17
Moisture	9.20	9.89	9.54	9.53	9.40	9.39	9.69	9.53
Energy (MJ/Kg)	17.35	17.68	17.81	17.70	17.13	17.68	17.85	17.19
Diet Cost (baht kg^{-1})	35.88	35.87	35.86	35.85	34.93	34.91	34.88	34.86

[a] 1 kg of Vitamin Mix consists of vitamin A 10,000,000 IU, D3 2,000,000 IU, E 1,500 IU, thiamine 2 gm, riboflavin 2.5 gm, pantothenic acid 14 gm, pyridoxine 2 gm, cyanocobalamin 10 mg, folic 0.5 gm, niacin 12 gm, K_3 2 gm, and C 20 gm.

[b] 1 kg of Mineral Mix consists of Ca 100,000 mg, P 80,000 mg, Cu 2,500 mg, Fe 1,200 mg, Mn 1,200 mg, Zn 1,540 mg, K 260 mg, I 740 mg, Mg 2,160 mg, Se 10 mg, and Co 240 mg.

Statistical analysis

The data were subjected to 1-way analysis of variance (ANOVA) and the difference between treatment means were multiple compared using Duncan's Multiple Range Test with significance predetermined at $P < 0.05$. Data on proportion were arcsine transformed [29] prior to analysis. The SigmaStat program version 3.5 was used for data analysis.

Results

Growth performances

The growth performances of juvenile Asian seabass were determined at 10th week. The final BW was 35.53 - 46.30 g, the TL was 13.46 - 14.66 cm, the specific growth rate (SGR) was 1.44 - 1.83 % day^{-1}, and the SR was 70 - 100 %. The BW and SGR in D2 - D4 were higher than D1 and there were significant differences ($P < 0.05$), whereas D5 - D8 were not significantly different ($P > 0.05$) from D1 (**Table 2**). The TL and SR did not show significant differences ($P > 0.05$) among the diet groups (**Table 2**). The results exhibited that FSM replacement up to 25 - 100 % of 10 % of protein from SM (D2 - D4) in juvenile Asian seabass diet caused increase in body weight and SGR when compared with control diet (D1).

Feed utilization

The FI of all groups was 48.59 - 50.93 g fish^{-1} and there were no significant differences ($P > 0.05$), while the FCR were 1.53 - 2.15 and the FCR of D2 - D4 were significantly decreased when compared with the other groups. The PER were 1.11 - 1.59 and presented a significant positive correlation with the FSM used levels, and D2 - D4 were significantly different ($P > 0.05$) from D1 and D5 - D8 (**Table 3**). The carcass percentages were 44.32 - 48.25 % and HSI were 1.90 - 2.30 % (**Table 3**), and there were no significant differences among groups. The feed capitals were 54.73 - 76.74 Thai baht per kg of fish (**Table 3**). D4 presented a significant lowest cost when compared with other diets ($P > 0.05$).

Table 2 Body weight and length, specific growth rate (SGR), and survival rate (SR) ± SE of Asian seabass affected by proportion of *Aspergillus niger* fermented soybean meal in the diets at 10th week.

Diet Groups*	Performances					
	Initial weight (g)	Final weight (g)	Initial length (cm)	Final length (cm)	SGR (% day^{-1})	SR (%)
D1	12.87 ± 0.04a	37.68 ± 0.08b	10.21 ± 0.05a	14.08 ± 0.38a	1.53 ± 0.01b	80 ± 5a
D2	12.88 ± 0.05a	45.39 ± 1.24a	10.32 ± 0.08a	14.66 ± 0.38a	1.79 ± 0.04a	95 ± 5a
D3	12.83 ± 0.04a	45.81 ± 2.66a	10.19 ± 0.07a	14.35 ± 0.47a	1.82 ± 0.09a	80 ± 0a
D4	12.90 ± 0.05a	46.30 ± 1.08a	10.18 ± 0.07a	14.51 ± 0.24a	1.83 ± 0.04a	85 ± 5a
D5	12.90 ± 0.05a	35.53 ± 0.84b	10.17 ± 0.06a	13.46 ± 0.48a	1.44 ± 0.03b	100a
D6	12.83 ± 0.04a	37.64 ± 1.79b	10.23 ± 0.07a	14.02 ± 0.45a	1.54 ± 0.07b	80 ± 5.77a
D7	12.82 ± 0.04a	38.19 ± 2.69b	10.18 ± 0.04a	13.60 ± 0.40a	1.55 ± 0.11b	70 ± 10a
D8	12.81 ± 0.03a	38.21 ± 1.97b	10.21 ± 0.06a	13.76 ± 0.46a	1.56 ± 0.07a	85 ± 5a

***** D1 - D4 are diets with 10 % of protein from soybean meal replaced by fermented soybean meal 0, 25, 75, and 100 %, respectively. D5 - D8 are diets with 20 % of protein from soybean meal replaced by fermented soybean meal 0, 25, 75, and 100 %, respectively. Different alphabet in the same column indicates significant statistical difference ($P < 0.05$).

Table 3 Feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), carcass percentage, hepatosomatic index (HSI), and feed capital ± SE of Asian seabass affected by proportion of *Aspergillus niger* fermented soybean meal in the diets at 10th week.

Diet Groups*	Performances					
	FI ($g\ fish^{-1}$)	FCR	PER	Carcass (%)	HSI (%)	Feed capital (Thai Baht kg^{-1})
D1	51.77 ± 0.19a	2.09 ± 0.02b	1.15 ± 0.01b	44.32 ± 1.04a	2.14 ± 0.15a	74.96 ± 0.63c
D2	54.16 ± 0.84a	1.67 ± 0.09a	1.45 ± 0.08a	47.77 ± 0.27a	2.30 ± 0.62a	59.95 ± 3.31ab
D3	53.39 ± 0.65a	1.61 ± 0.04a	1.51 ± 0.04a	48.25 ± 0.79a	1.90 ± 0.33a	57.82 ± 1.42ab
D4	50.93 ± 1.22a	1.53 ± 0.12a	1.59 ± 0.12a	47.75 ± 0.39a	2.06 ± 0.07a	54.73 ± 2.12a
D5	48.59 ± 1.69a	2.15 ± 0.05b	1.13 ± 0.02b	48.13 ± 1.51a	2.03 ± 0.29a	75.06 ± 1.58c
D6	54.12 ± 1.33a	2.20 ± 0.14b	1.11 ± 0.07b	45.90 ± 0.85a	2.22 ± 0.18a	76.74 ± 2.76c
D7	53.72 ± 1.62a	2.14 ± 0.17b	1.14 ± 0.0b	45.44 ± 1.16a	2.17 ± 0.06a	74.70 ± 2.96c
D8	51.43 ± 1.06a	2.04 ± 0.15b	1.21 ± 0.09b	46.51 ± 0.68a	2.15 ± 0.18a	70.94 ± 3.26bc

* D1 - D4 are diets with 10 % of protein from soybean meal replaced by fermented soybean meal 0, 25, 75, and 100 %, respectively. D5 - D8 are diets with 20 % of protein from soybean meal replaced by fermented soybean meal 0, 25, 75, and 100 %, respectively. Different alphabet in the same column indicates significant statistical difference ($P < 0.05$).

Discussion

4 % of protein from SM (10 % of total protein in diet) replaced by 25 - 100 % of protein from *Aspergillus niger* FSM presented significant improvement ($P < 0.05$) of BW, SGR, FCR, and PER of juvenile Asian seabass, while there was no effect on SR, carcass percentage, and HSI in this study. Cruz *et al.* [18] explained that the utilization of bacteria for feedstuff fermentation in appropriate conditions resulted in the reduction of antinutritional substances, and improved palatability as well as feed digest efficiency. During the fermentation process, bacteria produce various substances; acetic acid, lactic acid, propionic acid [14], etc. Lactic acid was able to destroy the antinutritional substances in soybean meal [14]. Likewise, the *A. oryzea* fermentation of SM reduced trypsin inhibitors and increased the number of small-size peptides, leading to an improvement of feed utilization [15]. The results of FM replacements by *Bacillus* spp. FSM in rainbow trout, *Oncorhynchus mykiss* [25], and red sea bream, *Pagrus major* [30], and utilization of *A. oryzea* FSM and *A. oryzea* directly mixed feed in parrotfish, *Oplegnathus fasciatus* [26] and olive flounder, *Paralichthys olivaceus* [31], showed higher growth rate and feed utilization than the control, while the survival rates were increased. Therefore, *A. niger* might have similar potential in SM fermentation, since it can yield functional substances; protease [15-17], cellulose and hemicellulases [19], lipase [20], phytase [21] and tannase [22], etc., during the process.

On the other hand, diets containing 8 % of protein from SM (20 % of total protein in diet); D5 and 25 - 100 % protein from FSM replaced diets (D6 - D8) in this study did not show significantly improved growth performances or feed utilization ($P > 0.05$) in juvenile Asian seabass; however, there was a trend for improvement. There might be a limited effect of soybean meal utilization in seabass. Tantikitti *et al.* reported that the replacement of FM by SM in Asian seabass should not exceed 10 % without effect on growth performance, while over this resulted in growth rate suppression [32]. Similarly, tests in other carnivorous marine fish such as pompano, *Trachinotus ovatus* [33], and orange-spotted grouper, *Epinephelus coioides* [34] showed that up to 10 % FM could be replaced by FSM without negative effects. Likewise, high levels of SM replacement affected performances in several fish species; decrease of feed efficiency (FE), PER, and growth rate in juvenile rockfish, *Sebastes schlegeli* [6], silver perch, *Bidyanus bidyanus* [35], and rainbow trout [7,8], while morphological abnormalities and bacterial community change of intestine were found in rainbow trout [8,9]. Drew *et al.* explained the reason of adverse growth performances with increased SM levels in the diet; SM is deficient in some essential amino acids (EAAs), methionine and lysine, which are important for fish growth [35]. Additionally, Azam and Lee reported that FSM was able to replace up to 40 %of FM protein in juvenile black sea bream, *Acanthopagrus schlegeli*, with supplementation of methionine, lysine, and taurine, without a negative effect on growth performance [36] Yang *et al*. demonstrated that the supplementation of methionine and lysine into 60 % of FSM replaced diet resulted in improved silver perch, *Bidyanus*, growth performance, while weight gain, FE, and PER declined in groups of fish fed higher levels of dietary FSM [37]. However, Martínez-Llorens *et al*. indicated that bigger fish seem to have higher tolerance to plant protein levels in diets than smaller fish [38], as the result of the FM being replaced by up to 40 % of FSM did not affect growth rate, FI, FE, PER, or survival rate of grower rockfish [6]. Moreover, the various abilities of plant protein utilization depends on different tolerance limits to antinutritional factors (ANFs) in plant proteins, which varies among species of fish [4]. The ability in the utilization of FSM as FM substitute in juvenile rockfish is lower than the ability of juvenile black sea bream under similar experimental circumstances [6].

Conclusions

The results of this study can conclude that the 10 % of total protein from SM protein in juvenile Asian seabass diet was able to substituted 25 - 100 % of *Aspergillus niger* FSM protein with effect on increase in BW, SGR, PER, and decrease in FCR, without adverse effects on SR, FI, carcass percentage, or HSI. Although it was attempted to increase the inclusion levels of SM protein up to 20 % of total protein in diet, including replacement of FSM protein in the diets, this did not improve the performances. Furthermore, when calculated, the feed capital per kg fish^{-1} from the FCR demonstrated that replacement of 10 % of SM protein by 25 - 100 % of FSM protein (D2 - D4) yielded almost 30 % lower cost than the others (D1 and D5 - D8), while the lowest was 100 % replacement (D4). This may suggest that 10 % of SM protein can replace 100 % of FSM protein in juvenile Asian seabass diet. Further study is needed to determine the available SM or FSM protein inclusion levels and EAAs addition in grower Asian seabass diet in order to reduce FM utilization, as well as improve product capital economization.

The indigenous *A. niger* from the aquaculture area of the Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, is an effective fungus for quality improvement of SM. *A. niger* FSM is an alternative protein source for growth and feed utilization improvement of Asian seabass. In addition, Asian seabass culturists can reduce feed cost by using FSM mixed feed and raise satisfactory income.

Acknowledgements

We thank all of our supporters. This study was financially supported by the Rajamangala University of Technology Srivijaya, Thailand, and the experimental facilities were supported by the Faculty of Science and Technology, Trang campus. Also, thanks to the technicians and students for their assistance.

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