

The evaluation of a functional feed additive derived from pineapple waste and downgrade crude extract for enhancing growth performance, feed utilization, and antimicrobial enhancement in Pacific white shrimp (*Litopenaeus vannamei*)

¹Rungkan Klahan, ¹Kitsana Krachabthong, ²Bundit Yuangsoi, ³Songsub Arungamol, ⁴Pinsurang Deevong, ⁵Jirapan Boonruang

¹ Department of Aquaculture, Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Thailand; ² Department of Fisheries, Faculty of Agriculture, Khon Khean University, Thailand; ³ Department of Fisheries, Faculty of Agricultural Technology, Sakon Nakhon Rajabhat University, Thailand; ⁴ Department of Microbiology, Faculty of Science, Kasetsart University, Thailand; ⁵ Phetchaburi Coastal Aquaculture Research and Development Center, Department of Fisheries, Thailand. Corresponding author: R. Klahan, supremrukiirun@gmail.com

Abstract. The aim of this study was to explore the technology for producing a functional feed additive from pineapple waste and degraded fruit to combat pathogenic microorganisms, particularly the *Vibrio* group, in white shrimp (*Litopenaeus vannamei*) and enhance production potential. Off-grade whole pineapples and edible-fruit by-products (peels and crowns) were used for crude extraction, and the pooled extract was subsequently dried by lyophilization (freeze-drying). To evaluate growth performance, feed utilization, health, and productivity of white shrimp, a feeding trial was conducted using feed supplements containing pineapple waste and degraded crude extract (PWDCE) produced via laboratory methods at different concentrations (0 as control, 250, and 340 ppt kg⁻¹ of feed) over a period of 90 days. The results showed that shrimp fed with PWDCE supplements at 250 ppt kg⁻¹ exhibited the highest growth rate, feed utilization, and cost-effectiveness compared to other groups ($p < 0.05$). Additionally, protease and chitinase activities were significantly lower in this group ($p < 0.05$). The study also assessed the non-specific immune system of shrimp supplemented with functional feed additives, finding no significant differences among groups ($p > 0.05$) in terms of total hemocyte count, phenol oxidase activity, superoxide anion production, and bacteria destroying activity. In conclusion, PWDCE at 250 ppt kg⁻¹ of feed, shows promise as a functional feed additive for enhancing white shrimp productivity and provide higher cost-effectiveness. However, its properties are not yet as clear as antibiotics.

Key Words: aquafeed, bromelain, gut microbiota.

Introduction. Pacific white shrimp, or white shrimp (*Litopenaeus vannamei*), farming is a crucial component of the global aquaculture industry; however, it faces numerous challenges, particularly from bacterial infections that can lead to substantial economic losses. One of the significant health issues that has long affected white shrimp farming is white feces syndrome (WFS), which is characterized by the appearance of white, floating fecal strings in shrimp ponds, indicating severe intestinal dysfunction. White feces syndrome (WFS) is a multifactorial disease that affects global shrimp production (Subash et al 2023). White feces syndrome in shrimp can result from various factors, including infections from pathogens such as white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV), yellow head virus (YHV), early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND), and gregarine parasites. Additional causes include waste accumulation in ponds, poor quality or deteriorated feed, and issues related to shrimp breeding, such as inbreeding, which can weaken shrimp and make them more susceptible

to infections. Furthermore, stress caused by various farming practices can reduce shrimp immunity, facilitating bacterial growth, and an imbalance of microorganisms in the digestive system due to antibiotic use can also contribute to white feces syndrome (Sudthongkhong 2017). Traditional methods to manage bacterial infections in shrimp farming, such as the use of antibiotics, have significant drawbacks, including the development of antibiotic resistance and residues in shrimp that pose risks to both the environment and human health. Consequently, there is a growing interest in natural and effective alternatives to antibiotics. To address these underlying causes—specifically those related to infection, inflammation, and nutrient absorption—pineapple *Ananas comosus* L. Merr. extract, which contains bromelain enzymes and saponins, has emerged as a promising treatment and preventive measure for white feces syndrome. Both of these active compounds aid in protein digestion, reduce tissue inflammation, and promote gut health while acting directly against some of the root causes of white feces syndrome. Research in aquatic animals has shown that saponins extracted from pineapple possess notable antimicrobial properties (Amelia et al 2020). Additionally, it has been reported that pineapple exhibits strong antimicrobial activity, which supports its use as an antimicrobial agent. The key antimicrobial compounds in pineapple—saponins and bromelain—function by interacting with cell membranes. Bromelain, in particular, acts as a proteolytic enzyme that can bind to bacterial cell membranes, causing damage and leading to bacterial cell death. The antimicrobial activity of these compounds has been demonstrated against both Gram-negative and Gram-positive bacteria, including multidrug-resistant *Pseudomonas aeruginosa* (Zharfan et al 2017). The antimicrobial effects of bromelain are primarily due to its ability to degrade bacterial cell walls and disrupt biofilm formation, which is a key defense mechanism used by bacteria to resist treatments and evade the host's immune response. Notably, a study by Zharfan et al (2017) found that pineapple extracts showed effective antimicrobial activity against *Pseudomonas aeruginosa*, with a minimum bactericidal concentration (MBC) of 0.75 g/ml.

Bromelain is well-known for its proteolytic capabilities, allowing it to hydrolyze protein molecules into smaller, more bioavailable peptides (Fennema 1996). Beyond its proteolytic function, bromelain also possesses immunostimulatory properties. It has been shown to activate white blood cells and modulate the immune responses of T cells and B cells in the blood, thereby enhancing the overall immune system function (Engwerda et al 2001). Additionally, bromelain exhibits anti-carcinogenic, anti-inflammatory, and antibiotic properties, which further contribute to its potential health benefits (Reddy et al 2013). Incorporating bromelain into shrimp feed not only helps reduce bacterial infections but also provides additional benefits, such as enhancing digestion and nutrient absorption, thereby improving overall shrimp health and growth performance (Klahan et al 2023). This dual functionality—combining antimicrobial action with digestive aid—makes bromelain an attractive feed additive for shrimp farming. Moreover, the use of natural enzymes like bromelain could mitigate the negative consequences associated with antibiotic use, such as the accumulation of residues in shrimp tissues and adverse ecological impacts. In an experiment conducted by Wiszniewski et al (2019) on juvenile sterlet (*Acipenser ruthenus*), it was observed that fish fed with bromelain-supplemented diets derived from pineapple showed higher total protein content, although levels of calcium, iron, copper, and zinc were reduced. Furthermore, bromelain supplementation led to an increase in the number of mucosal, enterocytes, and epithelial cells in the supranuclear zone, as well as enhanced activities of digestive enzymes such as lipase and pepsin. The study also found increased lysozyme activity, higher total protein and immunoglobulin levels, and elevated proliferation of T cells and B cells. However, no significant differences were observed in the activity of splenic macrophages or the potential killing activity of splenic phagocytes. Therefore, the aim of this research was to utilize the natural extracts also helps reduce the use of antibiotics, resulting in safe aquatic animals and reducing residues in the animals that affect exports. It also uses agricultural waste to respond to zero agricultural waste, increasing the value of safe food, as well as being environmentally friendly and safe for consumers.

Material and Method

Shrimp preparation. Twenty thousand Pacific white shrimp (*Litopenaeus vannamei*) at post-larval stage 12 (P12) were obtained from a private farm in Nakornpathom, Thailand. They were acclimatized in a 1,000-liter fiber pond with saltwater at a salinity of 10 ppt and fed a commercial pellet feed containing 38% protein five times a day for two weeks. The shrimp were then randomly selected and weighed for the experiment.

Enzyme preparation and specific activity assay. Pineapple waste and degraded crude extract (PWDCE) were obtained from "Batavia" pineapples (*Ananas comosus* L. Merr.) collected from a plantation in Ratchaburi province, Thailand. The waste pineapple components (peel and crown mixed) were derived from pineapples at the 1/3 dark green ripening stage, while degraded pineapples, which were smaller than normal and deformed, were also used and extraction followed the method of Klahan et al (2023). The crude extracts were then collected for specific activity assays according to the method of Ketnawa et al (2012) and subsequently dried using freeze-drying for feed preparation. The freeze-dried sample was dissolved in tap water at a concentration of 100 mg/mL, and the bromelain activity was measured again, including pH and TSS values, using a pH meter and TSS meter, respectively and prepared stock solutions for spraying on feed for the trial.

Feed trial preparation. Commercial sinking pellet feed for white shrimp containing 38% protein (Inteq®, Thailand) was supplemented with commercial bromelain and PWDCE at concentrations of 0, 250, and 340 mL/kg of feed. These treatments corresponded to 0, 250 ppt, and 340 ppt for both commercial bromelain and PWDCE. The enzyme stock solution was sprayed onto the feed, which was then fed to the shrimp immediately after preparation. The functional groups in the feed were analysed in the spectral range of 1020–3290 cm^{-1} using FTIR technique.

Experimental procedure. The experiment was conducted using a completely randomized design (CRD), with five treatments randomly assigned to four replicate groups of shrimp. The shrimp, with an initial weight of approximately 0.11 g, were stocked in glass tanks measuring 0.30 x 0.45 x 0.20 meters at a density of 60 shrimp per cubic meter. The study took place in the Department of Aquaculture Hatchery at the Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Phetchaburi, Thailand. This research was ethically approved by the Animal Ethics Committee for Experiments on Animals at Phetchaburi Rajabhat University, which follows the rules set by the Institute of Animals for Scientific Purposes Development (IAD) Thailand for the care of experiment animals, and issued the approval number IACUC PBRU230166005. There were five dietary treatments (T1–T5) for the shrimp, and each treatment had four replicates: T1 (control) — 40% crude-protein basal diet without additive; T2 basal diet plus commercial bromelain at 250 ppt kg^{-1} feed; T3 basal diet plus commercial bromelain at 340 ppt kg^{-1} feed; T4 basal diet plus pineapple-waste-and-off-grade crude extract (PWOCE) at 250 ppt kg^{-1} feed; and T5 basal diet plus PWOCE at 340 ppt kg^{-1} feed. The shrimp were fed the experimental diet five times daily over a 90-day period from January to March 2024. Throughout the experiment, daily records were kept of mortality and molting events, and the shrimp in each tank were counted and weighed monthly for growth rate (15 shrimps/replication; 60 shrimps /treatment) and feed utilization measurement.

Definitions for growth rate: W_i = initial mean weight (g shrimp^{-1}); W_f = final mean weight (g shrimp^{-1}); t = culture period (days); N_0 = number stocked (shrimp); N_f = number harvested (shrimp).

Formulas: weight gain (g shrimp^{-1}): $WG = W_f - W_i$; percent weight gain (%): $PWG = [(W_f - W_i) / W_i] \times 100$; average daily gain ($\text{g shrimp}^{-1} \text{ day}^{-1}$): $ADG = (W_f - W_i) / t$; specific growth rate: $SGR (\% \text{ day}^{-1}) = [(\ln W_f - \ln W_i) / t] \times 100$; survival rate (%): $SR = (N_f / N_0) \times 100$; total yield (g per tank) = $W_f \times N_f$;

Definitions for feed utilization and economics: t (days) = trial duration; N_0 = shrimp stocked; N_f = shrimp harvested; W_i (g shrimp^{-1}) = mean initial individual weight; W_f (g shrimp^{-1}) = mean final individual weight; B_i (g) = $W_i \times N_0$ = initial biomass; B_f (g) = W_f

$\times N_f$ = final biomass; H (kg) = $B_f/1000$ = harvested biomass; F_{cons} (g) = total feed consumed; F_{cons} (kg) = $F_{cons}/1000$; P_d = dietary crude-protein fraction (e.g., 0.40 for 40%); $N_{body,i} / N_{body,f}$ (g N shrimp⁻¹) = whole-body nitrogen per shrimp at start/end; kN (protein–nitrogen conversion) = 6.25; C_s (THB) = seed cost; C_{feed} (THB kg⁻¹) = feed price; P_{sale} (THB kg⁻¹) = selling price.

Fed utilization: total feed intake (g shrimp⁻¹): $TFI = F_{cons} / N$; feed intake (g shrimp⁻¹ day⁻¹): $FI = F_{cons} / (N \times t)$; daily feed intake (% body weight day⁻¹): $DFI = 100 \times (F_{cons} / t) \div [(B_i + B_f)/2]$; feed conversion ratio: $FCR = \text{feed intake/average daily gain}$; protein efficiency ratio: $PER = (B_f - B_i) \div (F_{cons} \times P_d)$; apparent net protein retention (ANPR) (%): $ANPR = 100 \times [(N_{body,f} \times N_f) - (N_{body,i} \times N_0)] \div [F_{cons} \times P_d / kN]$.

Economics: total cost (THB): $C_{total} = C_{feed} \times C_s$; income (THB): $Income = P_{sale} \times H$; B/C ratio: $B/C = \text{income} \div C_{total}$.

Water quality was monitored following standard methods. pH and temperature were measured daily (09:00 and 16:00 h) using a water quality multiparameter (HANAÒ, Thailand). Total ammonia nitrogen (TAN) was analysed weekly by the phenate (indophenol blue) colorimetric method (APHA 4500-NH₃) (APHA et al 2017). Nitrite-N (NO₂-N) was measured spectrophotometrically by the Griess diazotization method (APHA 4500-NO₂⁻ B) (APHA et al 2017). Calcium and magnesium were quantified weekly by EDTA titrimetric method following APHA 2340 C (total hardness as CaCO₃); calcium hardness was measured with murexide indicator (APHA 3500-Ca B), and magnesium was calculated by difference (total – calcium hardness). At the end of the study, the exoskeleton was separated to measure chitin content, and the edible flesh was analysed for proximate composition using the AOAC method (AOAC 2000).

Chemical composition and enzyme activity and chitin assay. At the end of the experiment, approximately 20 white shrimp from each treatment group were collected through random sampling. The shrimp were dried at 105°C for 2 hours in a hot air oven, then ground and subjected to proximate analysis according to AOAC methods (AOAC 2000). Moisture content was determined by oven-drying at 105 ± 2°C until a constant weight was achieved, with results reported on a wet basis. Ash is subjected to muffle-furnace incineration at a temperature of 550 ± 25°C until a constant weight is achieved. Crude protein is determined through Kjeldahl digestion using sulfuric acid and a catalyst, followed by distillation and titration to quantify total Kjeldahl nitrogen, which is then converted to protein by multiplying nitrogen content by 6.25. Crude fat is determined through Soxhlet extraction utilizing petroleum ether. Crude fiber is determined through acid-alkali digestion using the Weende method, followed by filtration, drying, and ashing until a constant weight is achieved. The protease and chitinase activities were analysed from enzymes extracted from the hepatopancreas of white shrimp following the method of Gimenez et al (1999), while chitinase activity was assayed by following the spectrophotometric method of Reissig et al (1955) and chitin content was assayed according to Khantaphant and Akkarachaneeyakorn (2017).

Measurements of immune parameters. For each treatment, 20 shrimp were sampled. Hemolymph (100 µl) was individually withdrawn from the ventral sinus of each shrimp (3rd walking leg) using a 1 ml sterile syringe with a 24-gauge needle and collected in an anticoagulant test tube containing trisodium citrate (30 mM), sodium chloride (0.34 M), and EDTA (10 mM). The hemolymph-anticoagulant mixture (diluted hemolymph) was used to measure total hemocyte count, phenol oxidase (PO) activity, superoxide anion (SO) activity, and bactericidal activity. Total hemocyte count (THC) was determined following the method of Yeh and Chen (2009). PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) according to the method of Söderhäll and Häll (1984). Superoxide anion production was quantified using the reduction of NBT (nitroblue tetrazolium) to formazan according to the method of Munoz et al (2000). Bactericidal activity was analyzed according to the method of Alderman and Smith (2001).

Research ethic approval. The procedures have been authorized by the Animal Ethics Committee for Experiments on Animals of Phetchaburi Rajabhat University and implemented in accordance with the experiment animal welfare regulation formulated by the Institute of Animals for Scientific Purposes Development (IAD) Thailand (Approval No. IACUC PBRU 230166005).

Statistical analysis. All experimental data, except for the FTIR spectrum, were analyzed using a completely randomized design with five replicates per treatment. One-way analysis of variance (ANOVA) was performed, followed by Duncan's multiple range tests, with a significance level set at $p < 0.05$.

Results. The results of the extraction properties and activities, including growth performance, feed utilization, and economic value including immune response are shown in the following tables and figures. The bromelain activity of PWDCE and commercial bromelain were significant difference among groups ($p < 0.05$) (Table 1).

Table 1

The activity and properties of bromelain crude extract

<i>PWDCE crude extract</i>	<i>Bromelain activity (mU/mg protein)</i>	<i>pH</i>	<i>Total soluble solid (ppm)</i>
Liquid type	88.95 ± 0.07 ^b	3.47 ± 0.15	1590 ± 42.43
Powder type	177.44 ± 10.51 ^a	3.83 ± 0.24	805.5 ± 27.58
Commercial bromelain	38.95 ± 3.13 ^c	4.48 ± 0.65	183.5 ± 40.31

Note: Means within a row with a different superscript letter are significantly different ($p < 0.05$).

The bromelain crude extract, both in liquid and powder forms, was analyzed for its activity and studied in relation to pH and TSS (total soluble solids), as shown in Figure 1. Both graphs demonstrated that the activity of bromelain in both liquid and powder forms did not correlate with either pH or TSS. Although it appears that the activity of bromelain in both forms may be influenced by pH, the results indicate that the pH of the enzyme crude extract cannot predict the enzyme's activity. This suggests that the stage of the pineapple, whether sweet or sour, does not impact the activity of the enzyme.

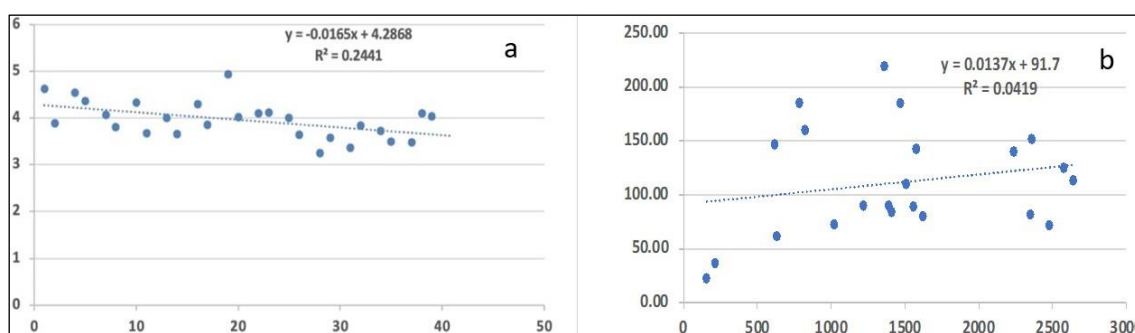


Figure 1. Regression analyses of (a) pH vs. bromelain activity and (b) total soluble solids (TSS) vs. bromelain activity for liquid and powder extracts; randomly selected crude bromelain extracts were used ($n = 27$).

Growth performance and feed utilization. The growth performance, represented by final weight, weight gain, average daily gain (ADG), specific growth rate (SGR), survival rate, percent weight gain, and yield, is presented in Table 2. The results showed no significant difference in final weight and weight gain ($p > 0.05$). However, weight gain, ADG, specific growth rate, and percent weight gain were highest in shrimp fed with PWDCE at 250 ppt kg^{-1} of feed ($p < 0.05$). The specific growth rate was lowest in shrimp fed with PWDCE at 340 ppt kg^{-1} of feed, while the survival rate improved for all shrimp fed with PWDCE-supplemented feed ($p < 0.05$). Additionally, shrimp fed with commercial bromelain at 340 ppt kg^{-1} of feed and PWDCE at 250 ppt kg^{-1} of feed showed higher yields than the other groups ($p < 0.05$).

Table 2

Growth performance, survival rate and yield of Pacific white shrimp fed with commercial bromelain and PWDCE for 90 days

Parameter	Treatments				
	T1	T2	T3	T4	T5
Initial weight (g)	0.11±0.01 ^a	0.11±0.01 ^a	0.11±0.01 ^a	0.11±0.01 ^a	0.12±0.01 ^a
Final weight (g)	6.49±0.16 ^a	7.28±0.39 ^a	6.87±0.84 ^a	7.68±1.16 ^a	5.31±1.01 ^a
Weight gain (g)	6.37±0.16 ^a	7.17±0.39 ^a	6.75±0.85 ^a	7.56±1.17 ^a	5.18±0.99 ^b
Weight gain (%)	5092.71 ±643.53 ^{ab}	6529.90 ±641.24 ^a	4993.77 ±408.11 ^{ab}	6621.81 ±1496.42 ^a	4003.97 ±251.39 ^b
Average daily gain (g/day)	0.08±0.01 ^{ab}	0.08±0.01 ^{ab}	0.08±0.01 ^{ab}	0.09±0.01 ^a	0.06±0.01 ^b
Specific growth rate (%/day)	4.96±0.10 ^a	5.11±0.12 ^a	4.99±0.35 ^a	5.02±0.28 ^a	4.52±0.07 ^b
Survival rate (%)	50.00±4.71 ^b	55.00±6.38 ^{ab}	66.66±6.67 ^a	60.66±6.67 ^a	66.66±6.67 ^a
Yield (g)	59.00±7.54 ^{ab}	65.50±3.53 ^{ab}	68.33±3.54 ^a	67.75±5.15 ^a	52.66±6.80 ^b

Note: Means within a row with a different superscript letter are significantly different ($p < 0.05$). T1 = control (0%), T2 = commercial bromelain 250 ppt kg⁻¹, T3 = Commercial bromelain 340 ppt kg⁻¹, T4 = pineapple waste and degraded crude extract 250 ppt kg⁻¹ and T5 = pineapple waste and degraded crude extract 340 ppt kg⁻¹.

Feed utilization and economic return were evaluated in terms of feed intake, feed conversion ratio (FCR), protein efficiency ratio (PER), production cost, income, and B/C ratio (Table 3). Shrimp fed with PWDCE supplemented at 250 ppt kg⁻¹ of feed showed outstanding feed utilization, with the lowest total feed intake, daily feed intake, and especially the lowest FCR ($p < 0.05$). This group also had the highest PER ($p < 0.05$). These results are consistent with the observed growth rate data.

Table 3

Feed utilization and economic return of Pacific white shrimp fed with commercial bromelain and PWDCE for 90 days

Parameter	Treatments				
	T1	T2	T3	T4	T5
Total feed intake (g)	16.68±1.44 ^b	18.21±1.95 ^b	21.80±2.05 ^a	10.87±1.18 ^c	21.80±2.05 ^a
Daily feed intake (%/day)	79.09±18.19 ^c	97.95±5.147 ^c	127.70±0.92 ^b	51.53±8.15 ^d	171.53±1.90 ^a
Feed intake (g/day)	0.22±0.04 ^a	0.23±0.01 ^a	0.26±0.02 ^a	0.13±0.01 ^b	0.26±0.02 ^a
Feed conversion ratio	2.24±0.51 ^{bc}	2.76±0.15 ^b	3.27±0.61 ^{ab}	1.45±0.24 ^c	4.33±1.03 ^a
Protein efficiency ratio	0.99±0.25 ^b	0.95±0.05 ^b	0.82±0.17 ^b	1.83±0.27 ^a	0.63±0.17 ^b
ANPR ¹ (%)	64.12 ± 2.50 ^b	86.51 ± 4.76 ^a	65.51 ± 0.29 ^b	87.11 ± 1.81 ^a	49.42 ± 4.67 ^c
Total cost ² (THB/kg)	13.52 ± 2.04 ^a	13.97 ± 0.77 ^a	15.76 ± 1.34 ^a	8.61 ± 0.77 ^b	15.76 ± 1.34 ^a
Income (THB) ³	5.90±0.75 ^{ab}	6.55±0.35 ^a	6.83±0.66 ^a	6.77±1.20 ^a	5.26±0.68 ^b
B/C ratio ⁴	0.44±0.06 ^b	0.45±0.02 ^b	0.43±0.05 ^b	0.78±0.05 ^a	0.33±0.05 ^c

Note: Means within a row with a different superscript letter are significantly different ($p < 0.05$). ¹ANPR = apparent net protein retention; ²total cost calculated from feed cost (feed intake) at 30 THB/kg feed and seed cost at 0.1 THB each shrimp; ³income calculated selling price at 105 THB (100 shrimps/kg); ⁴B/C ratio calculated from feed cost at 30 THB/kg and seed cost at 0.10 THB/shrimp and sale price at 105 THB/kg (100 shrimp/Kg).

The enzyme activity, in terms of protease and chitinase, was extracted from the hepatopancreas of shrimp fed with commercial bromelain and PWDCE-supplemented feed, and the chitin content in the exoskeleton is presented in Table 4, showing significant differences among the groups ($p < 0.05$). The highest activity was observed in shrimp fed

with PWDCE-supplemented feed at 250 ppt kg⁻¹, which is strongly related to feed and protein utilization and growth rate, following a consistent trend. Meanwhile, chitinase activity and chitin content in the exoskeleton appeared to align, with the highest chitinase activity and chitin content found in shrimp fed with commercial bromelain supplementation feed at 250 ppt kg⁻¹, followed by shrimp fed with PWDCE-supplemented feed at 250 ppt kg⁻¹ and the other groups ($p < 0.05$). This indicates that chitinase activity and chitin content are closely related, allowing for the prediction of the levels of both substances. Additionally, the data trend is consistent with growth rate observations.

Table 4
Protease specific activity (U/min/mg protein) and chitinase activity (U/ml) extracted from hepatopancreas of shrimp fed with commercial bromelain and PWDCE for 90 days (mean±SD)

Enzyme	Treatments				
	T1	T2	T3	T4	T5
Protease	3.47 ± 0.41 ^d	9.21 ± 0.77 ^b	8.12 ± 1.52 ^b	12.92 ± 0.75 ^a	5.29 ± 0.82 ^c
Chitinase	9.04 ± 0.55 ^b	15.44 ± 1.38 ^a	8.64 ± 0.63 ^b	9.84 ± 0.41 ^b	9.04 ± 0.84 ^b
Chitin (%)	6.82 ± 0.48 ^c	14.54 ± 0.81 ^a	7.98 ± 2.04 ^c	10.98 ± 0.86 ^b	7.50 ± 0.29 ^c

Note: Means within a row with a different superscript letter are significantly different ($p < 0.05$).

When focusing on the relationship between chitin content and chitinase activity, we found that they are indeed related. Figure 2 presents a regression analysis showing the relationship between chitin content and chitinase activity, with the percentage of chitin varying according to the chitinase activity. This demonstrates that the level of chitin and the enzyme's activity can predict each other.

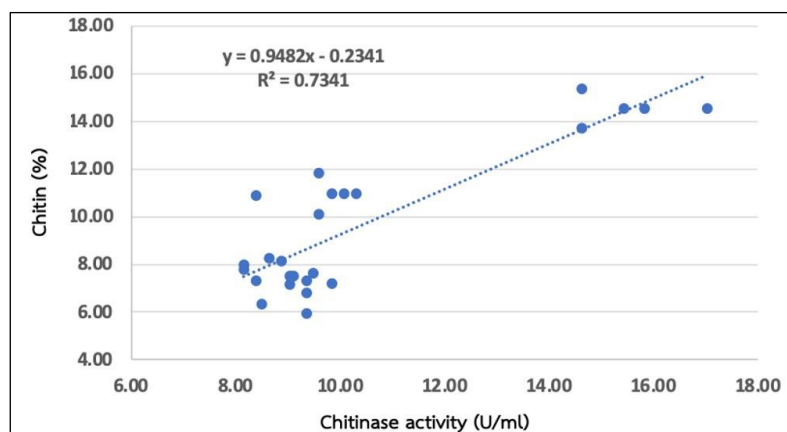


Figure 2. Regression of hepatopancreatic chitinase activity (U mL⁻¹) versus exoskeletal chitin content (%) in shrimp fed diets supplemented with commercial bromelain or pineapple-waste-and-off-grade crude extract (PWOCE); randomly selected shrimp (n = 25).

The edible flesh quality, as determined by proximate analysis of protein, fat, moisture, and ash content, is presented in Table 5. The protein content in the flesh was highest in shrimp fed with PWDCE supplementation at 250 ppt kg⁻¹ feed and lowest in the control group, which is the opposite of the moisture content, where the highest percentage was observed in the control group and the lowest in shrimp fed with PWDCE supplementation at 250 ppt kg⁻¹ feed ($p < 0.05$). The ash content was highest in shrimp fed with commercial bromelain supplementation at 340 ppt kg⁻¹ feed, and lowest in the control group and shrimp fed with PWDCE supplementation at 340 ppt kg⁻¹ feed ($p < 0.05$). The fat content in the flesh was similar across all groups ($p > 0.05$).

Table 5

Proximate analysis (%) of edible flesh of pacific whit shrimp fed with commercial bromelain and PWDCE for 90 days (mean±SD)

Proximate content (%)	Treatments				
	T1	T2	T3	T4	T5
Protein	84.59 ± 1.00 ^c	90.24 ± 2.21 ^{ab}	89.92 ± 1.69 ^{ab}	92.25 ± 0.97 ^a	87.05 ± 0.41 ^{bc}
Fat	2.40 ± 0.26 ^a	2.51 ± 0.35 ^a	2.36 ± 0.39 ^a	2.03 ± 0.06 ^a	2.05 ± 0.00 ^a
Moisture	7.32 ± 0.65 ^a	1.16 ± 0.07 ^c	1.10 ± 0.13 ^c	0.18 ± 0.03 ^d	6.54 ± 0.48 ^b
Ash	5.32 ± 0.20 ^c	6.01 ± 0.14 ^b	6.77 ± 0.59 ^a	5.85 ± 0.23 ^{bc}	5.42 ± 0.03 ^c

Note: Means within a row with a different superscript letter are significantly different ($p < 0.05$).

This research analyzed the functional groups in feeds (feed no. 901 and 903) that were digested with commercial bromelain and PWDCE, as shown in the FTIR peak graph (Figure 3). The results indicated that the functional group peaks were similar for either feed no. 901 or 903, regardless of whether they were digested with PWDCE or not. The molecular analysis results using Fourier transform infrared spectroscopy (FTIR) on white shrimp feed samples no. 901 and 903, both containing 35% protein, involved digestibility tests demonstrated in Figure 4. The FTIR technique identifies covalent molecules by detecting the vibration of bonds in organic compounds when exposed to infrared light. These vibrations result in peaks representing light absorption, which indicate specific molecular components. Key peaks identified during the study were linked to various organic compounds, such as C=O groups in amide and carboxylic ester bonds, with stretching vibrations observed in the ranges of 1650–1700 cm^{-1} and 1675–1750 cm^{-1} . Each experimental group showed consistent vibration patterns, highlighting similarities in their molecular structure. Specific peaks were found at 3290 cm^{-1} , 2927 cm^{-1} , and 2855 cm^{-1} , indicating the presence of functional groups such as NH_2 (amino acids), CH, and CH_2 (alkene stretching groups). These were notably prominent in the protein content of the feed, especially between soybean meal treatments. Further functional groups were detected at 1630 cm^{-1} , 1538 cm^{-1} , 1450–1404 cm^{-1} , and 1241–1021 cm^{-1} , with additional components like C=O in the Amide I band (1650), C=C unsaturated compounds (1610–1620), phenolic skeletons (1510), and silica (SiO_2) (1020). These FTIR results suggest similar structural characteristics across experimental feeds, although variations in functional group activity were observed between groups, potentially due to differences in protein treatment, such as heat or bond stability.

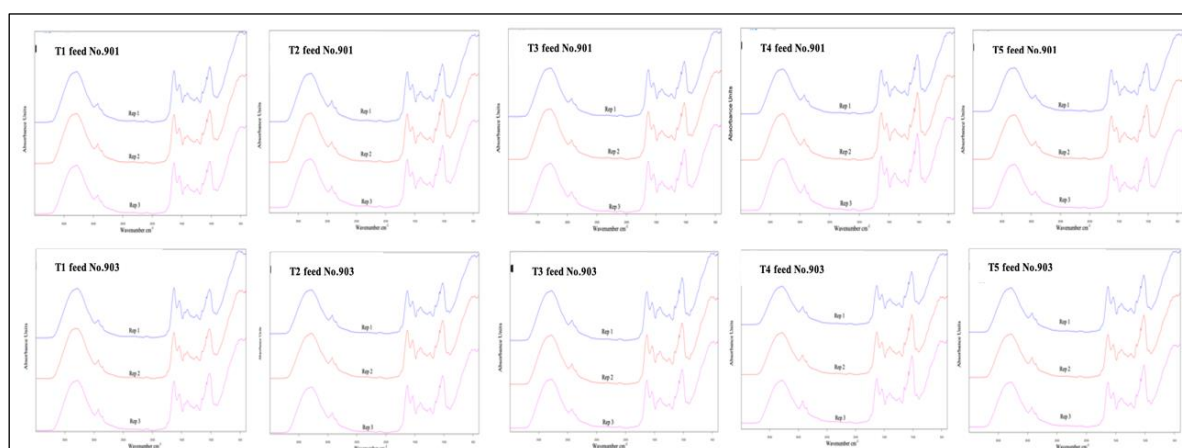


Figure 3. FTIR spectrum of feed digested with commercial bromelain and PWDCE. The 1st row is feed no. 901 and the 2nd row is feed no. 903.

The immune response data in term of total hemocytes count, phenol oxidase activity, superoxide anion production and bactericidal activity were not significantly different among groups ($p > 0.05$) (Table 6).

Table 6

Total hemocytes count, phenol oxidase activity, superoxide anion production and bactericidal activity of Pacific white shrimp fed with commercial bromelain and PWDCE for 90 days

Parameter	Treatments				
	T1	T2	T3	T4	T5
Total haemocytes count ($\times 10^5/\text{ml}$)	14.46 \pm 1.44	14.59 \pm 1.98	14.89 \pm 1.88	14.79 \pm 2.01	14.66 \pm 1.89
Phenol oxidase activity (U/mg protein)	183.45 \pm 21.42	178.97 \pm 16.94	179.82 \pm 14.44	184.42 \pm 17.71	181.02 \pm 10.42
Superoxide anion production	3.86 \pm 1.22	4.02 \pm 1.42	4.04 \pm 1.26	3.92 \pm 1.32	4.06 \pm 0.92
Bactericidal activity	1:4	1:4	1:4	1:4	1:4

Discussion. Based on the bromelain activity values, it was observed that drying bromelain enzymatic extracts resulted in higher enzymatic activity compared to their solution forms. This is likely due to freeze-drying, which minimizes protein loss and preserves the protein for a longer duration. Additionally, the concentration of the extract is greater in the dried form than in the aqueous solution. These findings align with the reports by Devakate et al (2009) and Nadzirah et al (2012), who found that freeze-drying pineapple juice resulted in bromelain powder with higher enzyme activity. Nadzirah et al (2012) demonstrated that bromelain powder from pineapple cork extract, which was acidic and lacked sucrose, showed higher activity than both pure extract and desalted forms.

The effects of using bromelain enzyme powder on the growth and feed utilization efficiency of white shrimp were also examined by incorporating bromelain enzyme powder (BL) into the experimental feed at concentrations of 250 and 340 ppt. These concentrations were chosen based on Klahan et al (2023), who found that liquid bromelain enzyme at 250 ppt had the most beneficial effect on white shrimp growth. In this experiment, when the feed was mixed with bromelain enzyme powder BL at 250 ppt, it yielded the best growth performance and feed utilization efficiency. This group of shrimps also exhibited higher yield, income, and benefit-cost (B/C) ratio than other experimental groups, while having lower production costs. These results correspond with the higher enzymatic activity observed in the bromelain powder extracts used in this group, suggesting a direct link between the enzyme's activity and shrimp growth performance. Bromelain, a crude extract from pineapple (*Ananas comosus*), is a mixture of thiol endopeptidases, along with other components such as phosphatases, glucosidases, peroxidases, cellulases, escharase, glycoproteins, and carbohydrates (Vasiljevic 2020). The various types of proteases in the bromelain extract are categorized into five groups: stem bromelain, fruit bromelain, pinguinain, ananain, and comosain, which are primarily extracted from the stem and fruits. According to Vasiljevic (2020), most protease enzymes in bromelain are derived from the stem, though other parts of the pineapple also contain some bromelain. The properties of this enzyme extract promote efficient protein digestion and nutrient utilization, requiring less feed while achieving higher growth rates. This leads to reduced energy loss during digestion and excretion, thereby enhancing energy availability for molting and growth. Furthermore, the bromelain extract contains saponin, which supports growth by improving the digestive system and immunity in shrimp. Saponin enhances nutrient absorption, contributing to better feed efficiency and growth in shrimp compared to other experimental groups (Klahan et al 2023). The combined effects of these substances help improve feed efficiency and growth performance in white shrimp, making this approach more effective than other feeding strategies. According to the study by Ketnawa et al (2012), the activity of bromelain and the protein content from various parts of pineapple waste—such as peel, core, stem, and crown—were examined in the Nang Lae and Phu Lae pineapple varieties. The results showed that different parts of pineapple waste, like the peel or crown, exhibited varying levels of protein digestion activity, with the highest activity found in the cap.

Bromelain is widely utilized in the aquatic animal feed industry due to its ability to digest proteins effectively. For instance, bromelain has been used in the hydrolysis of fish protein, producing fish protein hydrolysates with improved nutritional and antioxidant properties (Tanuja et al 2014). Additionally, bromelain enhances protein digestibility and utilization in aquatic animal feed, ultimately reducing feed costs (Arshad et al 2014). These properties suggest that extracts from subgrade pineapples, which use the entire fruit—including pulp, peel, and crown—yield a substantial amount of bromelain and other compounds, enhancing the overall efficiency of protein digestion, absorption, and utilization, leading to improved growth rates. However, the experiment revealed that using bromelain enzyme extract at a concentration of 340 ppt, both in commercial bromelain and bromelain derived from pineapple and converted into dry powder, resulted in lower feed and protein utilization efficiency and, consequently, lower growth in white shrimp compared to other experimental groups. This might be due to the presence of several types of protein inhibitors in the pineapple extract, which can negatively impact protein digestion and growth efficiency when bromelain is used in high concentrations (Tochi et al 2008).

Studies on the use of bromelain enzyme extracts in aquatic feeds have shown various benefits for different species. For example, Klahan et al (2023) found that using an aqueous bromelain enzyme extract at 250 ppt improved feed efficiency, protein utilization, and growth in white shrimp more effectively than other experimental groups. Similar studies have demonstrated that supplementing bromelain enzyme extracts in aquatic feeds can enhance protein utilization, growth, productivity, and overall health in species such as juvenile sterlet (*Acipenser ruthenus*) (Wiszniewski et al 2019), Mozambique tilapia (*Oreochromis mossambicus*) (Sharma et al 2021), Nile tilapia fingerlings (*Oreochromis niloticus*) (Gopalraaj et al 2022), eels (*Anguilla bicolor*) (Ulkhay et al 2023), and even amphibians like frogs, where bromelain enzyme supplementation has been shown to increase protein digestion efficiency and growth (Klahan & Sirithanawong 2015). Further analysis of the chemical composition of white shrimp meat revealed that shrimp fed with bromelain enzyme powder at 250 ppt had significantly higher protein accumulation in muscle tissue compared to other experimental groups ($p < 0.05$). This is attributed to improved protein digestion and utilization efficiency, as evidenced by increased protein content in shrimp tissues. The results indicate that bromelain enzyme powder is highly effective in promoting protein digestion, enabling shrimp to use and accumulate protein more efficiently, as seen in the higher protein percentage in their meat compared to other groups. Bromelain functions as a proteolytic enzyme that breaks peptide bonds at the carboxyl end of amino acids like arginine, tyrosine, or phenylalanine, rapidly digesting proteins such as casein and hemoglobin.

Interestingly, white shrimp fed with higher levels of bromelain enzyme extract demonstrated lower muscle protein accumulation, possibly due to incomplete or inefficient digestion of the excess protein, which could not be adequately absorbed. This excess protein burdened the shrimp's digestive system and metabolism, ultimately leading to reduced muscle protein accumulation (Yuan et al 2023). However, when examining intramuscular fat levels, it was found that shrimp across all experimental groups showed similar fat levels ($p > 0.05$). This is because most fat accumulates in the hepatopancreas, not in muscle tissue, and any excess nutrients not efficiently utilized by the shrimp are excreted. Consequently, the balanced digestion of both protein and energy nutrients does not significantly affect fat accumulation, consistent with findings from Kumar et al (2018) and Yuan et al (2023), who reported that protein levels did not affect fat accumulation in different animals. The moisture content in shrimp muscle was found to be inversely related to the protein content. The control group had a higher muscle moisture content than the other experimental groups ($p < 0.05$), followed by the groups supplemented with bromelain enzyme powder extracted from pineapple in both dry and commercial forms. According to Ou et al (2018), the muscle protein–water partition coefficient (KMP/w) is an important parameter for quantifying the potential for protein accumulation in muscle. A high protein accumulation in muscle results in a decrease in muscle water or moisture content, while a high moisture or water accumulation reduces protein accumulation. Additionally, a decrease in tissue water content may be related to an increased concentration of free amino acids (Huong et al 2010), which is also observed in other

marine crustaceans. The ash content was found to be highest in white shrimp fed with commercial bromelain enzyme powder at 340 ppt, while the control group and the group fed with dry-form bromelain enzyme powder extracted from pineapple at 340 ppt had the lowest ash content. For the ash content in white shrimp meat, the lowest value was observed in the control group and the group supplemented with dry bromelain enzyme extract at 340 ppt. However, the ash content was high in the group supplemented with commercial bromelain enzyme at 340 ppt, consistent with the chitin and chitinase enzyme levels in this group, which were low. This is related to the molting process of crustaceans, which involves accumulating chitin and minerals from the old shell and storing them in the hepatopancreas for use in molting and creating new shells. Thus, lower chitin and chitinase levels indicate slower molting compared to growth, resulting in the minerals prepared for molting being used for accumulation in the body or muscles, leading to higher mineral levels in the muscles than in other experimental groups.

In comparison, the shrimp group supplemented with dry bromelain enzyme extract at 340 ppt had lower chitin levels, chitinase enzyme activity, and mineral content in the muscles than the other three experimental groups, consistent with lower food utilization and growth values than the other groups. In crustaceans, before molting, shrimp store nutrients, including chitin and minerals from their shells, in the hepatopancreas. After molting, these nutrients are used to build new shells and support growth. The data indicate that shrimp receiving high levels of bromelain molt more effectively, requiring fewer stimulating factors from the minerals in the shell. The molting process in white shrimp depends on external factors such as temperature, nutrients, shrimp size, minerals, salinity, and environment, as well as internal factors like weight and hormones. Therefore, if shrimp receive sufficient nutrients, they will molt more frequently (Galindo et al 2009; Gimenez et al 1999). The chitin level indicates the accumulation of chitin for molting, but it depends on the completeness of the nutrients received, particularly protein and fat.

The accumulation of nutrients in the muscles of white shrimp and their growth were consistent with the protease activity, which was influenced by the enzyme extracts supplemented in the diet. The experiment found that the protease and chitinase activities extracted from the hepatopancreas of white shrimp varied depending on the form and level of bromelain enzyme extract in their diet. Protease activity was highest in the white shrimp group that exhibited the best growth and most efficient protein and feed utilization, specifically the group fed with a diet supplemented with 250 ppt of bromelain enzyme extract from dried pineapple ($p < 0.05$). Other experimental groups that received different levels of bromelain enzyme supplementation showed progressively lower protease activity, with the control group displaying the lowest activity ($P < 0.05$).

The increased production of protease enzymes suggests a high level of protein or short peptides in the diet, which stimulates the shrimp to produce enzymes proportional to the nutrients received (Klahan et al 2009). Although all experimental diets were of the same type, the key difference was the supplementation of bromelain enzyme. The diet supplemented with 250 ppt of dry bromelain enzyme extract stimulated the shrimp to synthesize more proteolytic enzymes, indicating that this level of enzyme supplementation effectively digested long peptides into shorter ones. This, in turn, prompted the shrimp to produce additional proteolytic enzymes to further digest the short peptides resulting from the initial protein breakdown by the bromelain enzyme extract. Bromelain, an exoprotease, improves protein digestion efficiency, leading to a higher quality and quantity of nutrients. A greater amount of nutrients from digestion can stimulate increased enzyme secretion in the shrimp's hepatopancreas (Zhou et al 2013). Additionally, bromelain, a plant protease, helps break down proteins in food into peptides and free amino acids, thereby supporting digestion and nitrogen utilization. It also positively influences the physiological conditions and growth of aquatic animals, such as fish (Abd Elnabi et al 2020; Li et al 2016). Protease activity is highly specific. For example, the most commonly used proteases in aquaculture feeds are neutral and alkaline proteases, such as alkaline serine endopeptidase (Chen et al 2021). It is important to note that the activity of proteases is highly specific in their cleavage patterns. For example, trypsin and alcalase are two proteases with different substrate specificities: trypsin targets peptide bonds located at the C-terminal side of lysine and arginine residues, while alcalase has broader specificity,

selectively hydrolyzing peptide bonds at the C-terminal side of hydrophobic residues (Vogelsang-O'Dwyer et al 2022). The specific cleavage sites of these enzymes affect the different types of short peptides produced, which in turn stimulate the body to synthesize more enzymes. Studies on enzyme supplementation have demonstrated that proteases in crustaceans, including shrimp and crabs, enhance protease enzyme activities.

Regarding the use of bromelain and commercial bromelain extracts supplemented in white shrimp feed to stimulate non-specific immunity, it was found that the measured values for total hemocyte count, phenol oxidase activity, superoxide anion production, and bactericidal activity were not statistically different across the experimental groups. For white shrimp and other crustaceans, the primary form of immunity is non-specific immunity, which responds to pathogens. The enzymes in the non-specific immune system can be categorized as either antioxidant or non-antioxidant enzymes, which are used to assess the functional status of the shrimp's immune system (Chakraborty et al 2021). In this experiment, the non-specific immune response of the shrimp was evaluated using total hemocyte count, phenol oxidase activity, superoxide anion production, and bactericidal activity. The results showed no statistically significant differences between the experimental groups. This could be due to the fact that the study did not involve a direct challenge with pathogens to test the shrimp's immune response, but rather focused on evaluating the health of shrimp fed with bromelain-supplemented feed. Therefore, the results may be inconclusive, even though bromelain has properties as a ligand (ions or molecules bonded to metal atoms). Bromelain is a unique bioactive proteolytic enzyme composed of cysteine proteases derived from pineapple (*Ananas comosus*) fruits and rhizomes. Cysteine promotes the formation of high-affinity metal-ligand complexes. Among the five cysteine amino acids that make up bromelain, two pairs are linked by disulfide bonds, while the remaining cysteine (Cys-25) is free and can function as a ligand. Bromelain has demonstrated strong antibacterial activity against both gram-positive and gram-negative bacteria, such as *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli*, *Chlamydia trachomatis*, and *Clostridium botulinum* (Mittal et al 2024). However, the concentration of bromelain or the types of pathogens present in the shrimp may not be optimal for bromelain's activity or the shrimp's immune system, resulting in no statistically significant differences in non-specific immunity values. Furthermore, according to Chakraborty et al (2021), bromelain acts as an antibacterial agent by inhibiting the growth of intestinal bacteria, such as *Vibrio cholerae* and *Escherichia coli*, and it also inhibits the production of enterotoxins by *E. coli* (ETEC). These bacteria may not match the specific pathogens encountered in white shrimp, leading to unclear differences in the immune responses among the experimental groups. The function of the immune system in crustaceans has been described by Cao and Cheng (2020), who reported that crustaceans produce many reactive oxygen intermediates (ROIs) during cell phagocytosis. When pathogens or foreign particles enter the host, they stimulate the host's NADPH-oxidase to produce various ROIs, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$) (Holmblad & Söderhäll 1999). These compounds can be directly toxic to pathogens (Roch 1999). The generation of superoxide ions, known as the respiratory burst, plays an important role in microbial killing. This phenomenon has been reported in various crustaceans, such as the crab *Carcinus maenas* (Bell & Smith 1993), the black tiger prawn *Penaeus monodon* (Song & Hsieh 1994).

Conclusions. The whole degraded pineapples and pineapple waste (peel and crown) can be used to extract bromelain enzymes. The appropriate concentration for using bromelain enzyme extract in dry powder form as a functional feed additive in white shrimp (*Litopenaeus vannamei*) feed to combat pathogenic microorganisms is 100 mg/ml. Mixing it with feed at a concentration of 250 ppt kg^{-1} of feed from pineapple waste and degraded crude extract (PWDCE) is considered the optimal level to significantly enhance the production potential of white shrimp and provide the highest cost-effectiveness. However, there is no clear evidence that bromelain, as an antibiotic or antimicrobial substance, has properties comparable to traditional antibiotics.

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Authors:

Rungkan Klahan, Department of Aquaculture, Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Thailand, 76000, e-mail: supremrukiirun@gmail.com

Kitsana Krachabthong, Department of Aquaculture, Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Thailand, 76000, e-mail: rukiirun@windowslive.com

Bundit Yuangsoi, Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand, e-mail: bundyu@kku.ac.th

Songsub Arungamol, Department of Fisheries, Faculty of Agricultural Technology, Sakon Nakhon Rajabhat University, Thailand, e-mail: songsub@kkumail.com

Pinsurang Deevong, Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand 10900, e-mail: pinsurang.d@kku.ac.th

Jirapan Boonruang, Phetchaburi Coastal Aquaculture Research and Development Center, Department of Fisheries, Thailand 76000, e-mail: supremrukiirun@gmail.com

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