The effect of feed supplement on growth, survival rate and immunity response of Pacific white shrimp Litopenaeus vannamei

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Abstract. An efforts to improve growth, survival and immunity response are still main issues in shrimp farming. The purpose of this study was to determine the effect of dietary supplements on the growth, survival and immunity response in order to increase phenotypic performance of Litopenaeus vannamei. The experiments were done by supplemented feed using i.e.,(A) Synthetic nucleotides (0.03 % of feed), (B) whole cell of Saccharomyces cerevisiae (100 mL/kg feed) and (C) Control (without feed supplement) and fed to the shrimp, Litopenaeus vannamei. The supplements were mixed in feed formulated and coated with chitosan, while the control -without feed supplement. The shrimp larvae were reared in 5 m3 tanks with two replications. The result showed that survival rate of shrimp that reared with supplemented synthetic nucleotide was 69.29% and with whole cell was 75.45% and control was 67.61%. Total haemocyte of shrimp before challenge test ranged from 287.2 to 465.7 x 10⁴ cells/mL, but after challenge test with WSSV, have shown different number of cell densities where in the treatments of synthetic nucleotide was 303.63 x10⁴ cell/mL, whereas in whole cell and control were 265 x 10⁴ cell / mL and 254.25 x 10⁴ cell / mL respectively. Immunity response of L. vannamei shrimp expressed from ProPO was significantly different between supplemented feed compared to control after challenged with WSSV, TSV and IMNV.

Keywords : Feed Supplement, Litopenaeus vannamei

1. Introduction
Shrimp is one of world fishery commodity and Indonesia is the country having huge natural resources which high potential to become leading country in producing shrimp from aquaculture. In 2005, Indonesia share value of 65 % from the total fisheries export [1]. At the present time, shrimp still become priority species for export due to the world demand of this species constantly high and stable.

Since year 2000, where Litopenaeus vannamei started introducing from Hawaii and Florida (USA) to Indonesia, the production of this species significantly increased while the production of Penaeus monodon decreasing sharply. The total production of shrimp was 350,000 MT/year [2]. Most of the improved genetically broodstock of L. vannamei are imported from USA, and seed production of
its is very developed and even in Indonesia most of the hatchery able to produce shrimp fries/post-larvae in huge number. Therefore, culture of *L. vannamei* become sustain in Indonesia.

The price of imported broodstock is quite expensive although with certificate of Specific Pathogen Free (SPF), but cannot be guaranty that will not attack by diseases especially various virus such as WSSV, TSV, IHNV and IMNV, or do not resistant to environmental change and some time slow growth.

Several efforts have been done to prevent an infection of diseases caused by viruses, bacteria, fungus and parasite by applying immune-stimulant in hatchery and grow-out farms. Immuno-stimulant that was use is beta glucan as an alternative prevention [3];[4];[5];[6]. A positive result of using immune-stimulant has shown an increasing response of non specific immunity in shrimp.

Instead of immune-stimulant, nucleotide as food supplement, also able to enhance immunity and growth of shrimp. The usage of nucleotide for crustacean and fish culture seriously concern in recently. [7]. mentioned that nucleotide is a structure of RNA, DNA and cofactor. The chemical component of nucleotide consisted of heterocyclic sugar, one or more than one compounds of phosphate. In general, nucleotide basis is derivate from purine and pyrimidin, while compound of sugar are pentosa (5-sugar carbon) deoxyribosia or only ribosa.

In the cell, nucleotide having an important function in preparation of energy, metabolism, and signaling, therefore nucleotide is become essential for supporting all function in the body of organism. Nucleotide gave a natural building block for cellular growth, cell multiplication, biosynthesis protein and enzyme. By using nucleotide will act as internal ribosoma entry site (IRES), therefore a mediator for translation. Some literatures mentioned that by using nucleotide in rearing of fish and crustacean could improve metabolism, recovery from diseased, accelerate immune respond, reduce mortality and parasite infection, increase stress tolerant, improve reproduction and feed conversion ratio could improve immune, metabolism, and enzyme. By using commercially nucleotide (Nucleo-20) for rearing of *L. vannamei* in pond could improve growth of 13.5 %, survival 4.03% and FCR reduce up to 10.6 % [6] and resistant to WSSV [7].

On the other hand, crustacean has no adaptive immune response with immunoglobulin [17]; [18]. Recently, there is an interesting phenomenon that named quasi-immune response have been reported against to WSSV in *P. japonicus* [19].

Research on seed and broodstock productions of *L. vannamei* using nucleotide in *L. vannamei* for increasing immunity response to virus and bacteria’s diseases become important to produce seed and broodstock of *L. vannamei*, that immune to diseases. The present study aimed to produce *L. vannamei* that immune to diseases by using feed supplement.

2. **Methods**

The research were done in two steps, that are larval rearing and producing prospective broodstock

2.1. **Assessment of feed supplement in larval rearing of Pacific white shrimp *L. vannamei***

In the larval rearing experiment of *L. vannamei*, three treatments were held by feed supplement, these are supplemented by synthetic nucleotide, whole cell and control. The feed supplemented by synthetic nucleotide at ratio of 0.03%, consisted of from Uridine-5 monophosphate disodium salt, cytidine-5 monophosphate disodium salt, guanosine-5 monophosphate disodium salt, adenosine-5 monophosphate disodium salt, inosine-5 monophosphate disodium salt. While whole cell of *S. cerevisiae* was produce by culturing with growth media of yeast at 2 L volume. Each of supplement was mixed in microdiet and coating with chitosan, and in control (without supplement) only add by chitosan.

Larval rearing of *L. vannamei* was held in hatchery of the Research Institute for Mariculture Gondol – Bali until post larvae-12 (PL-12) stage. The larvae were detected their diseases status, the
larvae must be free from any virus of TSV, WSSV and IMNV. The viruses were analysis by speedy PCR. The larvae were reared in 5 m^3 with 2 replications

The larvae were fed with Chaetoceros ceratophorum, Thallasiosira sp, Skeletonema sp., Artemia sp. and micro diet, All feed were added with synthetic nucleotide and whole cell S cerevisiae as a treatment and control without supplement, The larvae were fed 3–4 times a day. At initial stage the larvae were fed C. ceratophorum at density of 10,000-30,000 cell/mL, and followed by Thallasiosira sp dan Skeletonema sp at density of 25,000-30,000 cell/mL then Artemia sp. at density of 5-15 nauplii/larvae. The water was exchanged between 15-50% from zoea to post larvae stages. The larvae development, survival and health condition were observed every day. Biosecurity was applied strictly during larval rearing periods,

2.2. Assessment of feed supplement in rearing of prospective broodstock of Pacific white shrimp L.vannamei

The prospective broodstock were fed by artificial diet supplemented by synthetic nucleotide and whole cell S.cerevisiae and compare to control without supplement. The prospective broodstock were reared by flow-through system and fed twice a day at amount of 2.5-5.0% of biomass. The growth rate were observed and at the end of experiment the histology of gastrointernal were observed and immunity respond were evaluated.

2.3. Immunity response

Observation of immunity response were held by cohabitation challenge test to WSSV, IMNV and TSV viruses. The chalenge test were done in aquarium at volume of 60x40x39.5 cm aqul volume of water at 94.8 L. Each aquarium were kept 40 individuals of prospective broodstock with 2 time replication. Initial body weight of shrimp 21.67 ± 1.59 g. Fresh food that infected by viruses, were fed twice a day base on the treatments. The challenge test were held for 96 hours. The hemocyte and immune response expressed by pro PO were observed at the end of experiment.

Haemolymph were collected from each shrimp from ventral-sinus cavity using nidle 25 gauge and syringe 1 mL containing antiocoagulant solution. Anti coagulant solution that was used is modified from K-199 solution [20];[21] to become KC-199 by adding 2.38g/L HEPES and 5% L-cystein. The calculation of total hemocyte were done by taking 0.1 mL hemolymph randomly from treated shrimp with 0.4 mL KC-199 solution and mixed gently, and hemocyte were counted by hemocytometer under light microscope 400 x.

The proPO analyses were done through preparation of Hemocyte Lysate Supernatant (HLS). Preparation of HLS by taking 0.1 mL of hemolymph from shrimp and mixed with 0.4 mL KC-199 solution and centrifuse at speed of 2,500 rpm for 10 minute at temperature of 4°C. Hemocytes were washed and gathering in cold cacodylate (CAC) buffer at pH of 7.0 ( 0.01 M natrium cacodylate, 0.45 M NaCl, 10 mM CaCl_2, 26 mM MgCl_2). This solution was homogenized and centrifuge at speed of 2,500 rpm for 20 minutes at temperature of 4°C. HLS result was quickly used as enzyme source.

Activity of phenoloxidase on HLS was counted by spectrophotometer using L-3,4-dihydroxyphenylalanin (L-DOPA) as a substrate and trypsin as elisitor. At amount of 200 μl HLS was incubated at 200 μl 0.1% trypsin on CAC buffer at room temperature for 30 minutes then add of 200 μl L-DOPA (0.3% in CAC buffer). After reaction mixed then dilute by 600 μl CAC buffer and measure by 490 nm wave length. Result of measurement was compare with standard CAC buffer, L-DOPA and Trypsin for controlling oxydation substrate spontaneously. One unit enzyme activity designated as protein content in HLS which is mesured by standard bovine serum albumin as standard protein.

3. Result and Discussion

The result showed that survival among the three treatments did not show any differences, the value of survival for treatments by synthetic nucleotide was 69.29 %, whole cell 75.46 % and control 67.61 % respectively. The survival of each stage for all treatments are shown in Figure 1. Most of the
mortality caused by cannibalism and vibrioses bacteria in the rearing water. Vibrio is opportunistic pathogen as an agent of disease in shrimp larvae.

**Figure 1.** Survival rate of *L. vannamei* shrimp reared with different feed supplements during experiment.

The prospective broodstock were produce by continuing rering the larvae started at PL34. The larvae were reared in circular tank at capacity of 8 m$^3$ with flowthru system. The density of the larvae for each treatment was 1,000 ind./tank. The result revealed that the growth of shrimp fed by supplemented syntetic nucleoted feed was 24.93 g, whole cell *S. cerevisiae* 24.47 g and control 21.67 g. There was no significat different in growth rate among the treatments (Figure 2).

**Figure 2.** Body weight (A) and body length (B) of prospective shrimp broodstock *L. vannamei* cultured with different treatments in order to improve immunity response.

Based on histological results of intestine (gastrointestinal) of the prospective broodstock for each treatment show that the size of microvilli gastrointestinal was varied between two treatments (synthetic nucleotide and whole cell) mainly on high and wide of microvilus, while for control the size of microvilli was relatively uniform and a bit shorter (Figure 3). This variation sizes of gastrointestinal were related to the metabolism in which caused by supplemented feed that fed to the shrimp, or most probably caused by increment of size (high) of villus and thicknes also increasement number of villus cell and expanded surface area of intestine mucosa as an impact of suplemented feed. [13].
Figure 3. Performance microvillus intestinal of prospective shrimp broodstock *L. vannamei* with different feed supplement (A) synthetic nucleotide, (B) Whole cell of *S. cerevisiae* and (C) Control

In the challenge tests with WSSV for 96 hours have shown that *L. vannamei* fed on supplemented feed with synthetic nucleotide more immune, the only 1 ind. (2.5%) shrimp was dead while in the supplemented whole cell dead shrimp was 5 ind. (12.5%), while in the control dead shrimp was 13 ind. (32.5%). The mortality of shrimp that challenged by WSSV is presented in Table 1. Looking at the number of dead shrimp seems that the WSSV character is pathogenic virus, and the candidate broodstock of shrimp fed with supplemented nucleated synthetic is more immune to WSSV infection.

In the challenge tests of shrimp by IMNV, most of shrimp were immune with IMNV. This can be seen from the mortality very low. The mortality occured only 2 ind. (5%) in the shrimp fed with supplemented feed by whole cell of *S. cerevisiae* and in control only 2.5 % mortality. In the challenge test of shrimp by TSV, the mortality of 3 % occurred in the shrimp fed with supplemented feed by synthetic nucleotede, whole cell 0 % and control 2.5% (Table 1) and seems that prospective broodstock of shrimp cahallenged by IMNV and TSV having immune response.

The different of mortality among treatments most probably related to immune response that caused by supplemented fed. Some researcher mentioned that by using nucleotide supplement in crustacean feed have shown positive impact of metabolism, improve resisitency to diseases, accelerate immune response, reduce mortality due to parasite infection and enhance stress tolerance. [12];[13].

Table 1. Mortality of prospective broodstock of Pacific white shrimp *L. vannamei* after challenge tested with WSSV, IMNV and TSV

| Treatment         | WSSV      | IMNV      | TSV       |
|-------------------|-----------|-----------|-----------|
|                   | Mortality (%) | Mortality (%) | Mortality (%) |
|                   | Time (hours) | Time (hours) | Time (hours) |
| Synthetic Nucleotide | 0 24 48 72 96 | 0 24 48 72 96 | 0 24 48 72 96 |
| Whole Cell       | 0 0 0 0 0  2.5 0 0 0 0 7.5 0 0 2.5 0 0 |
| Control          | 0 0 0 0 0 15 32.5 0 0 0 0 2.5 0 2.5 0 0 0 |
The results of calculations on the number of hemocytes (Table 2) from prospective shrimp broodstock *L. vannamei* after challenged with WSSV, showed that the administration of synthetic nucleotide (303.63 x 10⁴ sel/mL) was higher than control (254.25 x 10⁴ sel/mL), whereas feed supplemented with whole cells *S.cerevisiae* gave relatively low number of hemocytes (165 x 10⁴ sel/mL).

The number of hemocyte in the prospective broodstock, *L. vannamei* after challenged with IMNV and TSV was similarly. Result of hemocyte number after challenged with IMNV showed varying values between treatments. On synthetic nucleotide supplements, the total hemocytes tend to be same each 367.63 x 10⁴ cells/mL. The number of hemocytes in the control (348.13 x 10⁴ sel/mL) was lower compared to whole cells treatment, 644.38 x 10⁴ sel/mL.

Some scientists argue that hemocyte are indicator of nonspecific defense mechanisms against infection from pathogenic organisms in invertebrates. Hemocyte activity will function actively at all ages of shrimp and is used to determine shrimp health and evaluate the cellular immune response in crustaceans.

**Table 2.** Total number of hemocytes cells after challenge tested with WSSV, IMNV and TSV

| Treatment        | Initial | WSSV  | IMNV | TSV  |
|------------------|---------|-------|------|------|
| Synthetic Nucleotide | 465.7   | 303.63| 367.63| 847.5 |
| Whole cell       | 357.2   | 165   | 644.38| 1058.13 |
| Control          | 287.2   | 254.25| 348.13| 955   |

In crustaceans, haemocyte is involved in immediate defense reactions such as modulation, encapsulation, and phagocytosis [22];[14];[23]. The presence of cell circulation has implications for different immune responses, such as melanization and coagulation mediated by the release of hemocyte effectors, such as prophenoloxidase (proPO), activating systems, tansglutaminase or anti microbial peptides. Besides immune-related protein, shrimp can be described as clotting protein, lysozyme, LPS agglutinine and β-glucan-binding protein. The proPO system activation process requires protease participation and this action requires precise control to prevent tissue damage.

The observation results on phenoloxidase activity of prospective broodstock *L. vannamei* which cultured with dietary supplement and Control (without dietary supplement) and have been challenged WSSV, IMNV and TSV are shown in Figure 4. The analysis using three substrate buffer (L-DOPA, Trypsin dan CAC) to control substrate oxidation spontaneously, enzyme activity in the Hemocyte Lysat Supernatan (HLS) can be detected. Phenoloxidase is produced from the activation of the prophenoloxidase (proPO system) as a cascade phenomenon with several stages for the performance of organ functions.

It shows that the prospective broodstock has been challenged with WSSV on the CAC substrate can be seen high value of proPO activity (0.0128 - 0.0134) on diatery supplemented of synthetic nucleotide and whole cells of *S.cerevisiae*, while in the control is 0.0122 unit/menit/mg protein. ProPO activity on shrimp that has been challenged with IMNV similar value on all of three substrate. While, the shrimp were challenged with TSV, the highest proPO activity was obtained in the CAC substrate.
Figure 4. Value of ProPO on prospective broodstock of Pacific white shrimp *L. vannamei* after challenge tested with WSSV (A), IMNV (C) and TSV (D) in three substrates (CAC, Trypsin and L-DOPA)

The immune mechanism of shrimp is unlike as fishes that have immunoglobulins system. Shrimp immunoglobulin is known as Pro-phenoloxydase Activating Enzyme (PPA) (Soderhall dan Cerenius 1992). PPA is a protein located in granular hemocyte cells and can be activated by lipopolysacharida (LPS) and β 1,3-Glucan, which can stimulate prophenoloxidase to phenoloxidase. As a result of these changes will produce a kind of Opsonin Factor protein that can induce agranular hemocyte cells to carry out the process phagocytosis [24]. Hemocyte cells also degranulate and some proteins are released as a immune response [25].

**Conclusion**

The use of feed supplements with form of synthetic nucleotide or whole cells of *S cerevisiae* produces a significant effect on growth, immune response and performance of the digestive intestine on Pacific white shrimp *L. vannamei*.

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