Jurnal Biosains Pascasarjana Vol. 25 (2023) pp
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BIOENCAPSULATION ARTEMIA WITH *Bacillus subtilis* AND SODIUM ALGINATE ON TOTAL HEMOCYTE AND SURVIVAL RATE OF *Litopenaeus vannamei* INFECTED WITH *Vibrio parahaemolyticus*

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Abstrak

Pencegahan penyakit pada budidaya udang dapat dilakukan dengan meningkatkan respon imun udang dalam melawan penyakit. Salah satunya ialah dengan penggunaan imunostimulan. Bahan imunostimulan yang dapat digunakan adalah B. subtilis dan Natrium alginat yang terbukti mampu meningkatkan respon imun udang. Tujuan penelitian ini adalah untuk mengetahui pengaruh bioenkapsulasi Artemia dengan kombinasi B. subtilis dan Natrium alginat terhadap total hemosit dan kelangsungan hidup post larva udang vaname yang terinfeksi V. parahaemolyticus. Penelitian ini menggunakan Rancangan Acak Lengkap (RAL) dengan 5 perlakuan dan 4 ulangan. Total hemosit dan kelangsungan hidup diukur pada H0, H7 (tujuh hari pasca pemberian bioenkapsulasi), H9 (satu hari pasca infeksi), dan H15 (tujuh hari pasca infeksi). Hasil penelitian ini menunjukkan bahwa total hemosit H7 dan H9 pada perlakuan P3 berbeda nyata ($p<0.05$) terhadap perlakuan lain. Kelangsungan hidup udang vaname yang diberikan bahan imunostimulan, menunjukkan P3 memiliki tingkat kelangsungan hidup tertinggi dan berbeda nyata ($p<0.05$). Kesimpulan dari penelitian ini adalah bioenkapsulasi Artemia dengan kombinasi B. subtilis dan Natrium alginat dapat meningkatkan total hemosit dan kelangsungan hidup post larva udang vaname yang terinfeksi V. parahaemolyticus.

***Kata Kunci**: Bacillus subtilis, Bioenkapsulasi Artemia, kelangsungan hidup, natrium alginat, total hemosit*

Abstract

Disease prevention in shrimp farming can be done by increasing the shrimp's immune response to disease. One way is to use immunostimulants. Immunostimulants that can be used are B. subtilis and sodium alginate which are proven to increase the immune response of shrimp. This study aimed to determine the effect of bioencapsulation of Artemia with the combination of Bacillus subtilis and sodium alginate on total hemocyte and the survival rate of white shrimp post-larvae infected with V. parahaemolyticus. This study used a completely randomized design (CRD) with 5 treatments and 4 replications. Total hemocytes and survival rate were measured at H0, H7 (seven days post-bioencapsulation), H9 (one-day post-infection), and H16 (seven-days post-infection). The results of this study showed that the total hemocytes H7 and H9 in the P3 treatment were significantly different ($p<0.05$) from other treatments. The survival of white shrimp treated with immunostimulants showed that P3 had the highest survival rate and was significantly different ($p<0.05$). The conclusion of this study is that the bioencapsulation of Artemia with the combination of B. subtilis and sodium alginate can increase total hemocyte and the survival rate of white shrimp post-larvae infected with V. parahaemolyticus.

Keywords: Bacillus subtilis, Bioencapsulation Artemia, survival rate, sodium alginat, total haemocyte count

1. INTRODUCTION

White shrimp is one of the essential commodities in local and export markets, so the production of white shrimp is expected to continue to increase. The volume of shrimp production in 2020 is more than 900 thousand tons. The target in 2024 is 2 million tons (KKP, 2021). The high demand for white shrimp encourages intensive cultivation.

DOI 10.20473/jbp.v25i1.2023.8-14

Intensification can be negative effect, one of which is bacterial disease, mostly caused by *Vibrio* spp. (Kurniawinata *et al.,* 2021).

Strategies to prevent disease in shrimp are carried out by using antibiotics, but antibiotics can have a negative impact, namely causing accumulation of harmful residues and developing resistant pathogens (Singer *et al.,* 2019), so that immunostimulants can be an innovative approach to shrimp health management because it can increase the immune response of shrimp to pathogenic microorganisms (Barman *et al.,* 2013).

There are many immunostimulants that can be used, such as bacteria, algae derivatives, animal derivatives, nutritional factors, and hormones/cytokines (Barman *et al.,* 2013). One type of bacteria that can be used as an immunostimulant is *Bacillus subtilis*. *Bacillus subtilis* is a gram positive bacterium. The bacterial cell wall is composed of peptidoglycan. Peptidoglycan is a bacterial polymer that can enhance the shrimp's immune response (Pan *et al.,* 2015).

The addition of *B. subtilis* to white shrimp infected with the pathogen *V. Parahaemolyticus* shows that *B. subtilis* can increase the immunity of white shrimp (Vogeley *et al.,* 2019). Latest research shows that using *Lactobacillus lactis* alone is less able to protect organisms from pathogens. The best result that can increase the immune response is the use of a combination of *L. lactis* bacteria and sodium alginate (Loh *et al.,* 2021) so that in this study a combination of bacteria and sodium alginate will be used.

Sodium alginate can be used as an immunostimulant because it has been shown to increase the immune response of shrimp (Santos *et al.,* 2019). Alginate found in the cell walls of brown algae consists of salts of calcium, magnesium, potassium, and sodium alginate (Rasyid, 2005). Alginate is found in Sargassum's cell wall, a polysaccharide type with immunomodulatory activity. The polysaccharide content can activate the response immune non-specific in white shrimp (Santos *et al.,* 2019).

The method of giving anti-microbial agents to shrimp can be done in various ways, one of which is bioencapsulation. Bioencapsulation is an effective method to provide antimicrobial compounds to shrimp post larvae (Sivagnanavelmurugan *et al.,* 2015). Bioencapsulation is the delivery of a substance into a living organism which is then used as food for the target organism. Live organisms that can be used as vectors for the distribution of antimicrobial materials are Artemia (Santos *et al.,* 2019).

Artemia is a crustacean that is used as the best natural live food that can be stored and used in the hatchery process. Artemia has a non-selective filter feeder that can absorb all particles in the maintenance media. Bioencapsulation of Artemia with immunostimulant gives a good effect by increasing the stimulation of the immune response in white shrimp post-larvae (Rudtanatip *et al.,* 2019).

The distribution of antimicrobials through Artemia bioencapsulation has been widely carried out (Sivagnanavelmurugan *et al.,* 2015; Rudtanatip *et al.,* 2019; Santos *et al.,* 2019; Loh *et al.,* 2021). Bioencapsulation of Artemia with a combination of *B. subtilis* and Sodium alginate for white shrimppost larvae has not been carried out, so this study needs to be carried out to determine the effect of bioencapsulation of Artemia on total hemocytes and survival rate on white shrimp post-larvae infected with *Vibrio parahaemolyticus.*

2. RESEARCH METHOD

2.1 Sodium Alginate Extract and Culture of *B. subtilis* and *V. parahaemolyticus*

Sodium alginate was extracted from *Sargassum polycystum* seaweed taken from Talango, Madura. The seaweed was dried and sodium alginate was prepared according to the method of Setyoaji *et al.,* (2019).

B. subtilis was isolated from white shrimp pond sediments based on the method of Truong *et al.* (2021). Bacterial isolates of *B. subtilis* was inoculated on Tryptic Soy Agar (TSA) media and incubated at 30ºC for 24 hours. The colonies formed were then cultured on Tryptic Soy Broth (TSB) media and incubated at 30ºC for 24 hours. Population density using a spectrophotometer at 600 nm (Mirbakhsh *et al.,* 2021).

V. parahaemolyticus was isolated from the hepatopancreas of white shrimp infected with *V. parahaemolyticus*, streaked on Thiosulfate Citrate Bile Salts Sucrose (TCBS) and incubated at 37ºC for 24 hours. Single green colonies were cultured on TSB media and incubated at 30ºC for 24 hours. Population density using a spectrophotometer at 600 nm (Fu *et al.,* 2017).

2.2 Bioencapsulation of Artemia

Commercial *A. franciscana* cysts from Great Salt Lake Utah, USA. Artemia cysts were hatched in seawater for 24 hours (instar II) (Widodo *et al.,* 2016). The bioencapsulation of Artemia process was carried out for 8 hours in a beaker glass with a density of 15 nauplii/mL (Loh *et al.,* 2021). The bioencapsulated doses were sodium alginate 0.4 g/L (Immanuel *et al.,* 2012) and 10^8 CFU/mL *B. subtilis* (Vidal *et al.,* 2018).

2.3 White Shrimp Post Larvae Treatment

White shrimp post larvae (PL 8) came from hatcheries in Sidayu, Gresik, Indonesia. Maintenance of white shrimp post larvae at a density of 5 shrimp/Liter (Sivagnanavelmurugan *et al.,* 2015). Feeding pellets (200-300 µm) were carried out three times a day at 07.00, 12.00, and 17.00 ad libitum during the rearing period. Feed treatment with Artemia bioencapsulated was given at 14.00 as many as 60 nauplii/larvae/day (SNI, 2009) for seven days of treatment.

On day 8th, white shrimp post larvae were challenged with *V. parahaemolyticus* by immersion method at a density of 10^6 CFU/mL LD_{50} (Garcia-Bernal *et al.,* 2018). Immersion was carried out for 2 hours and then transferred to clean seawater, while in treatment (K-) only water was changed (Joseph *et al.,* 2015).

2.4 Research Design

The research method used is an experimental method using a Completely Randomized Design (CRD) with 5 treatments and 4 replications.

In this study, the treatments used were K- (Artemia without bioencapsulation and without infection), K+ (Artemia without bioencapsulation and *V. parahaemolyticus* infection), P1 (Artemia bioencapsulation with *B. subtilis* and infection), P2 (Artemia bioencapsulation. with sodium alginate and infection), P3 (bioencapsulation of Artemia with the combination of *B. subtilis* and sodium alginate and infection).

2.5 Total Hemocyte Count

Total hemocytes in white shrimp post larvae were calculated based on the whole shrimp body. White shrimp post larvae was put into a 1.5 mL effendorf tube containing 0,4 mL of anticoagulant (Trisodium citrate, NaCl, EDTA, aquades). The white shrimp post larvae was ground and homogenized (Tampangallo *et al.,* 2018), then the suspension was taken using a pipette and dripped on a hemocytometer. Performed under a light microscope with a magnification of 400x. Total hemocytes were calculated based on Abdollahi-Arpanahi *et al.* (2018).

2.6 Survival Rate Count

The survival rate of white shrimp post larvae was observed until day 16th, then the survival rate was recorded at the beginning of

rearing (H0), after treatment (H7), one day after infection (H9), and seven days after infection (H15). The survival rate calculations based on Loh *et al.* (2021).

3. RESULTS AND DISCUSSION

3.1 Total Hemocyte

There are three types of total hemocytes in shrimp, namely hyaline, granular and semi-granular. In shrimp, hemocytes are key in the immune mechanism. Hemocyte cells can activate prophenoloxidase, phagocytic activity, encapsulation, and nodule formation (Madani *et al.*, 2018).

Based on the results of the Analysis of Variance (ANOVA) showed that the administration of the combination of *B. subtilis* and sodium alginate had an effect on increasing the total hemocytes of white shrimp post-larvae ($p<0.05$). Total hemocytes on H0 were not significantly different ($p>0.05$) from the other treatments. On H7 after treatment with Artemia bioencapsulation and on H9 after being challenged with *V. parahaemolyticus* bacterial infection showed that P3 was significantly different ($p<0.05$) to the other treatments. On H15 after seven days of infection showed that K+ was significantly different ($p<0.05$) from the other treatments The total hemocytes of white shrimp post larvae was on Table 1.

Table 1. Total hemocytes of white shrimp post larvae

Treatment	Total Hemocyte (10^6 cell/mL)			
	H0	H7	H9	H15
K-	6.38 ± 0.21^a	6.42 ± 0.17^c	6.50 ± 0.19^b	6.53 ± 0.24^b
K+	6.34 ± 0.12^a	6.42 ± 0.07^c	4.98 ± 0.19^c	5.51 ± 0.42^c
P1	6.38 ± 0.18^a	7.50 ± 0.41^b	6.46 ± 0.07^b	6.49 ± 0.05^b
P2	6.33 ± 0.17^a	7.34 ± 0.22^b	6.26 ± 0.11^b	6.39 ± 0.08^b
P3	6.31 ± 0.15^a	8.33 ± 0.78^a	7.18 ± 0.22^a	6.94 ± 0.33^a

Table 1, H0 is the initial hemocytes of white shrimp post larvae which ranged from 6.31 x 10^6 cell/mL to 6.38 x 10^6 cell/mL. After being given Artemia bioencapsulation treatment up to H7, it showed an increase in total hemocytes compared to the control treatment. This indicates that the shrimp's immune response increases. Increased immunity can be caused by the peptidoglycan content in the cell wall of *B. subtilis* bacteria (Ramadhani *et al.*, 2015) and the polysaccharide content in sodium alginate from *S. polycystum* (Santos *et al.*, 2019).

Peptidoglycan and polysaccharides can work as Pathogen Related Molecular Patterns (PAMPs) which are molecules that can induce innate immunity in shrimp. The introduction of PAMPs by the shrimp immune system creates the necessary solutes to trigger both humoral and cellular responses in shrimp (Maliwat *et al.*, 2021).

On H9 and H15 after *V. parahaemolyticus* infection, showed the lowest total hemocytes in shrimp that were not given immunostimulants. The decrease in total hemocytes is a form of shrimp immune response to pathogens. Hemocyte cells migrate to bacteria-infected organs (Pudgerd *et al.*, 2021) then hemocyte cells will carry out a phagocytosis process that begins with attachment, ingestion, digestion, and destruction of microbes (Chifdhiyah, 2012). Hemocyte cells will be damaged and lysis after the phagocytosis process is complete so that the number of hemocytes will decrease (Song *et al.*, 2003).

3.2 Survival Rate

The survival rate is a comparison value between the number of living organisms at the end of rearing and the

number of organisms at the beginning of rearing, which is expressed in percent. Based on the results of the Analysis of Variance (ANOVA), showed that the administration of a combination of *B. subtilis* and sodium alginate had an effect on the survival rate of post-larvae white shrimp ($p<0.05$). Survival on H0 and H7 was not significantly different ($p>0.05$) from other treatments. The highest survival was at H9, namely, the K- treatment but not significantly different ($p>0.05$) from the P3 and P2 treatments, while at H15, the P3 treatment was the best treatment with an 89% SR which was significantly different ($p<0.05$) to all treatment.The survival rate of white shrimp post larvae is shown in Table 2.

Table 2. Survival rate of white shrimp post larvae

Treatment	Survival Rate (%)			
	H0	H7	H9	H15
K-	100 ± 0^{a}	100 ± 0^{a}	$98 \pm 1,6^{a}$	$95^{a} \pm 2,6^{a}$
K+	100 ± 0^{a}	100 ± 0^{a}	$78 \pm 6,7^{c}$	$66^{d} \pm 6,9^{d}$
P1	100 ± 0^{a}	100 ± 0^{a}	$92 \pm 3,7^{ab}$	$81^{bc} \pm 5,3^{c}$
P2	100 ± 0^{a}	100 ± 0^{a}	$90 \pm 3,7^{b}$	$80^{c} \pm 1,6^{c}$
P3	100 ± 0^{a}	100 ± 0^{a}	$96 \pm 3,7^{ab}$	$89^{b} \pm 2,6^{b}$

Table 2. The survival rate on H7 shows 100% results so the administration of Artemia bioencapsulation can increase the survival of white shrimp. On H9 and H15 after being infected with *V. parahaemolyticus* there was a decrease in the survival rate of white shrimp post larvae. The highest survival rate was in the P3 treatment using Artemia bioencapsulation with a combination of *B. subtilis* and sodium alginate. The high survival rate of vaname shrimp postlarvae is due to the polysaccharide content in the sodium alginate and the peptidoglycan content in *B. subtilis* can stimulate the shrimp's immune system by increasing resistance to disease (Pan *et al.,* 2015; Santos *et al.,* 2019).

5. CONCLUSIONS AND SUGGESTIONS

Bioencapsulation of Artemia with the combination of 10^{8} CFU/mL *B. subtilis* and 0,4 g/L sodium alginate can increase the total hemocytes and the survival rate of white shrimp post larvae infected with *V. parahaemolyticus.*

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