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Quality of Dried *Bacillus* NP5 and its Effect on Growth Performance of Tilapia (*Oreochromis niloticus*)

[1]Diah Ayu Satyari Utami, [2]Widanarni and [2]M. Agus Suprayudi
[1]Aquaculture Program, Gradute School, Bogor Agricultural University, Bogor, 16680, West Java, Indonesia
[2]Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Bogor, 16680, West Java, Indonesia

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Corresponding Author:
Widanarni,
Department of Aquaculture,
Faculty of Fisheries and Marine Sciences,
Bogor Agricultural University,
Jalan Agatis, Dramaga Campus,
Bogor, 16680, West Java, Indonesia

ABSTRACT
The main things that need to be considered in the preparation of probiotics are viability during preparation and storage which are the disadvantages of the use of fresh culture probiotics. Dried probiotic can be applied through the feed, easy to be applied and has a long shelf life but application of dried probiotic in aquaculture is still not widely studied. This study aimed to evaluate the quality of dried *Bacillus* NP5 as the probiotic through *in vitro* assays and determine the best dose for the growth performance of tilapia. The treatment of *in vitro* assays including the production of dried probiotic without using of the coating material and dried by spray drying method (NS); freeze drying method (NF); with using of the coating material and dried by spray drying method (WS); freeze drying method (WF). The treatment which showed the best result at *in vitro* assays was applied for *in vivo* assays. The *in vivo* assays containing 4 treatments and 5 replicates which were control (K) and the administration of dried *Bacillus* NP5 Rf^R (10^{10} CFU g^{-1}) in feed with dose of 0.5% (A), 1% (B) and 2% (C). The fish fed 3 times a day by at satiation for 28 days. Probiotic that encapsulated by maltodextrin and dried by spray drying method that stored in room temperature had the higher percentage product, viability after drying process and storage. The administration of 0.5% dried *Bacillus* NP5 showed the best growth performance in tilapia.

Key words: Dried, *Bacillus* NP5, growth, tilapia

INTRODUCTION

Tilapia (*Oreochromis niloticus*) is a commodity that is widely cultivated in the world because has rapid growth, wide tolerance of environmental condition and has high market demand. Developing countries, especially Asia such as China, Indonesia, Philippines and Thailand are major producers of tilapia (Josupeit, 2005). In 2012, the production of tilapia by the aquaculture sector in Asia increased to 3.3 million tons (FAO., 2014). The production increased in order to fulfill the increasing market demand for tilapia.

Fulfillment of the high market demand for tilapia is reached through the intensive culture application. Artificial feed is one of the main components in intensive culture. Feed is the energy source for the fish to grow and takes the biggest part of production cost in aquaculture in the amount of 40-89% (Suprayudi, 2010). Therefore, the important aspects that have to be considered in the feeding management is how to increase the feed efficiency and fish health status (Naylor *et al*., 2009). It is necessary to produce the feed which not only gives high growth performance but also results a higher survival rate and lower feed conversion ratio, so that the nutrients in the feed are utilized optimally and wasted to the environment in a little amount.

Probiotics are live or dead microbes or microbial components that give some benefits to the host (Fuller, 1989; Lazado and Caipang, 2014). The development of research on the use of probiotics in aquatic animals are the result of the

necessity of eco-friendly aquaculture system (Gatesoupe, 1999), in which the main role of probiotics in aquaculture include the increase of fish productivity and feed utilization, disease control, water quality control and bioremidiation of the polluted environment, so that the application of probiotics can be the answer to the efficiency of feed utilization and eco-friendly aquaculture system. The main things that need to be considered in the preparation of probiotics are viability during preparation and storage which are the disadvantages of the use of fresh culture probiotics (Wang *et al*., 2008). Dried probiotic can be applied through the feed and has several advantages such as the easier application and a long shelf life (Decamp and Moriarty, 2007). The previous study showed that the application of freeze-dried form of *Lactobacillus rhamnosus* JCM 1136 could increase the Lactic Acid Bacteria (LAB) number in the intestine and phagocytic activity of rainbow trout (Panigrahi *et al*., 2005). In addition, application of dried probiotic in aquaculture is still not widely studied. One of the factors that affect the probiotic performance is dose (Nayak, 2010). This study aimed to evaluate the quality of dried *Bacillus* NP5 as the probiotic through *in vitro* assays and determine the best dose for the growth performance of tilapia.

MATERIALS AND METHODS

Preparation of dried probiotic and *in vitro* assays: Probiotic used in this study was *Bacillus* NP5 isolated from the gastrointestinal tract of tilapia (Putra, 2010). Probiotic was given the rifampicin resistant marker (*Bacillus* NP5 Rf^R). The method to construct the marker was carried out according to Widanarni *et al.* (2004). Colonies of *Bacillus* NP5 Rf^R were cultured in luria bertani slant agar.

The treatment of *in vitro* assays including the production of dried probiotic without using of the coating material and dried by spray drying method (NS); freeze drying method (NF); with using of the coating material and dried by spray drying method (WS); freeze drying method (WF). The fresh culture probiotic (inoculant) was harvested by centrifugation at speed of 7000 rpm for 20 min to obtain the probiotic biomass. Furthermore, the probiotic biomass (pellet) was homogenized using a homogenizer in Phosphate Buffered Saline (PBS) for the uncoating treatments and sterile solution of 10% maltodextrin for the coating treatments. The proportion of inoculants with PBS or sterile solution of 10% maltodextrin is 1:1 (v/v). Furthermore, those were dried using a spray dryer with an inlet temperature of 120°C and an outlet temperature of 70°C for drying treatment by spray drying method and a freeze dryer with a temperature of -50°C for drying treatment by freeze drying method. The dried forms of probiotic were measured their product percentages and viabilities after drying process. The dried forms which had the high product percentages and viabilities were stored at Room Temperature (RT) and Cold Temperatures (CT) for a month, then tested their viabilities and observed their physical qualities after storage. The treatment which showed the best result at *in vitro* assays was applied for *in vivo* assays.

Product percentages and viabilities after drying process: The product percentage of probiotic after drying process was calculated by comparing the amount of inoculant which would be dried with dried forms which produced after drying (v/w). Viability of probiotic was calculated by comparing the total probiotic before and after drying. Total probiotic was enumerated by the spread plate technique using LBA (Luria Bertani Agar) that was supplemented with 50 mg mL^{-1} rifampicin (LBA+Rf).

Viabilities and physical qualities of probiotic after storage: Viability of probiotic was calculated by comparing the total probiotic after drying and storage. Total probiotic was enumerated by spread plate technique using LBA+Rf. The physical quality of probiotic after storage was observed by observing the form and color of dried forms of probiotic after drying and the changes in the form and color after storage for a month.

Experimental design of *in vivo* assays: The fish strain used in this study was tilapia nirwana strain that were obtained from Center of Fish Breeding Research Sukamandi, West Java, Indonesia. The fish were acclimatized in the tanks sized 120 L. The 6.38±0.05 g fish were reared in aquarium sized $60\times30\times30$ cm^3 at a density of 10 fish per aquarium.

This study was conducted in Completely Randomized Design (CRD) consisting of 4 treatments with 5 replications, including administration of dried *Bacillus* NP5 in feed with different doses (0.5% (A), 1% (B) and 2% (C)) which were 10^{10} CFU g^{-1} as the concentration and control (K) without administration of dried *Bacillus* NP5. The test feed was commercial pellet (Hi-Provite 781-1) with 30.18% protein content, 5.25% fat, 52.53% carbohydrate, 9.12% ash and 2.92% crude fiber. The dried *Bacillus* NP5 was added to the feed and mixed with 2% egg white as a binder. The fish were fed three times a day (08.00, 12.00, 16.00) by at satiation for 28 days. Replacement of water in the rearing tanks as much as 80% of total volume were conducted every 4 days to maintain water quality. The water quality was maintained in the normal range for freshwater culture, according to Boyd (1990) that dissolved oxygen >5 mg L^{-1}, temperature at 24-30°C, pH at 6.5-9.5 and Total Ammonia Nitrogen (TAN) <0.52 ppm.

Observation of growth performance parameters: The growth performance parameters including Survival Rate (SR); Specific Growth Rate (SGR); Feed Conversion Ratio (FCR) were calculated with the following equation:

$$SR(\%) = \frac{Nt}{N0} \times 100$$

$$SGR(\%) = \frac{\ln We - \ln Ws}{t} \times 100$$

$$FCR = \frac{F}{Bt - B0}$$

where, Nt is the number of fish that live at the end of the study (individuals), N0 is the number of fish at the beginning of the study (individuals), We is the average weight of fish at the end of the study (g), Ws is the average weight of fish at the beginning of the study (g), t is the duration of the study (day), F is the amount of consumed feed (g), Bt is the biomass of fish at the end of the study (g), B0 is the biomass of fish at the beginning of the study (g).

Enumeration of total viable bacteria count and total *Bacillus* NP5 Rf^R count in the fish intestine: The enumeration was carried out by the spread plate technique. The fish intestine was taken as much as 0.1 g and homogenized with 0.9 mL of 0.85% physiological buffer solution. Furthermore, there were the serial dilutions of the sample and then spread them as much as 0.05 mL on LBA medium for Total Viable Bacteria Count (TVBC) and LBA+Rf medium for total *Bacillus* NP5 Rf^R count (TNP5) in the intestine. The enumeration of TVBC and TNP5 were conducted at the beginning and end of the study.

Statistical analysis: All data was tabulated using Microsoft Excel 2007. The data of *in vitro* assays were analyzed by descriptive analysis using table and graphic while the data of *in vivo* assays were analyzed by one way-ANOVA, then continued by Duncan test with significance as much as 0.05 using SPSS 20.

RESULTS

***In vitro* assays:** The higher product percentages after drying were shown by the treatments which using the coating material which dried by spray drying method (WS) and freeze drying method (WF) (5.18±0.98; 4.79±0.64%), while the other treatments without using the coating material (NS and NF) resulted the lower product percentages were 0.40±0.00; 0.81±0.01%, respectively (Fig. 1a). The higher probiotic viabilities also were shown in WS and WF which were 99.88±0.12; 94.59±3.47%, respectively while NS and NF showed the lower viabilities were 63.84±6.97; 81.48±10.49%, respectively (Fig. 1b).

Based on data of Fig. 1a and 1b, so the viability test after storage and physical quality observation of dried forms of probiotic were only conducted in the best treatments (WS and WF) which were stored in Room Temperature (RT) and Cold Temperature (CT). After being stored for a month, the highest probiotic viability was obtained in WS which stored at room temperature (WSRT) with a percentage of 92.54±2.03% and followed by WSCT, WFCT and WFRT (61.76±0.04; 56.87±0.12; 37.43±2.99%) (Fig. 2).

At the end of the *in vitro* assays, there were the form changes in WSCT and WFRT which there were an clotting or caking and discoloration while WSRT and WFCT were able to maintain their physical forms which were in powder and flake form (Table 1).

Based on the data of *in vitro* assays, the production method of dried probiotic (*Bacillus* NP5) and storage method that chosen for *in vivo* assays was WSRT. This treatment had the higher product percentage and viability after drying, the

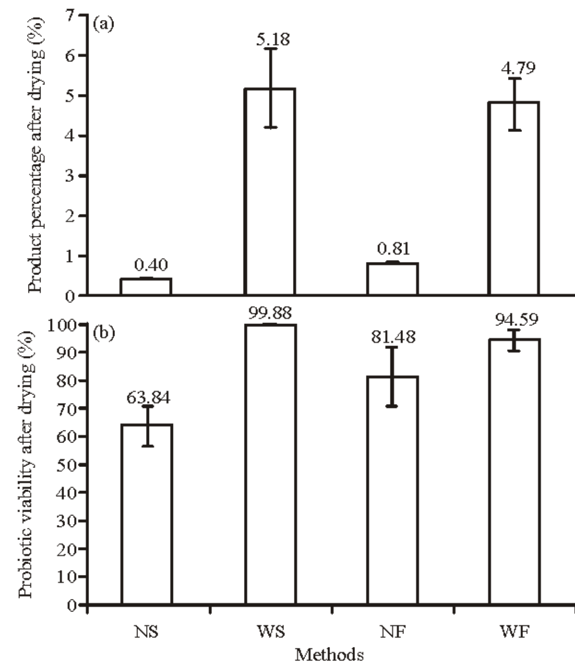

Fig. 1(a-b): (a) Product percentage and (b) Probiotic viability after drying. Production dried probiotic without using of the coating material which dried by spray drying method (NS); freeze drying method (NF); with using of the coating material which dried by spray drying method (WS); freeze drying method (WF)

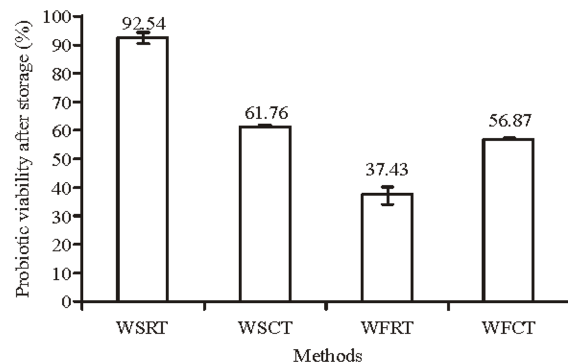

Fig. 2: Probiotic viability after storage. Production of dried probiotic with using of the coating material which dried by spray drying method and stored in room temperature (WSRT); cold temperature (WSCT); dried by freeze drying method and stored in room temperature (WFRT); cold temperature (WFCT)

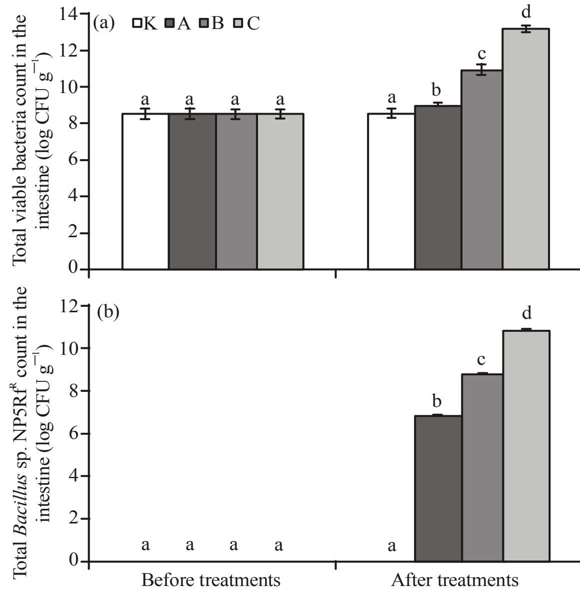

Fig. 3(a-b): (a) Total viable bacteria and (b) *Bacillus* NP5 Rf[R], count in the intestine of tilapia before and after treatments. Different letters on each bar on the same observation period (Mean±SD) indicated significant differences (Duncan, $p<0.05$). Control (K), administration of dried *Bacillus* NP5 in feed at a dose of 0.5% (A), 1% (B), 2% (C)

Table 1: Physical quality of dried probiotic

Treatments and storage method	Physical form	
	Initial	Final
WS		
RT	Powder	Powder
	White	White
CT	Powder	Clot and watery
	White	White yellowish
WF		
RT	Flake	Caramelized clotting or caking
	Transparent	Golden brown
CT	Flake	Flake
	Transparent	Transparent

WS: Coating material spray drying, WF: Coating material freeze drying, RT: Room temperature, CT: Cold temperature

Table 2: Survival Rate (SR), Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) of tilapia

Parameters	Treatments			
	K	A	B	C
SR (%)	100±0.00[a]	100±0.00[a]	100±0.00[a]	100±0.00[a]
SGR (%)	2.72±0.12[a]	3.04±0.05[b]	2.89±0.17[ab]	2.75±0.14[a]
FCR	1.49±0.09[b]	1.25±0.07[a]	1.50±0.01[b]	1.47±0.12[b]

Different letters in the same row indicated significant differences (Duncan; $p<0.05$), the values shown were means and standard deviations

higher viability after storage and could maintain its form as well as its initial form after being stored for a month.

***In vivo* assays:** The survival rates of tilapia in this study were 100±0.00% in all dose treatments. The best growth performance was shown by A with the higher Specific Growth Rate (SGR) was 3.04±0.05% which was significantly different ($p<0.05$) with control (K) and C (2.72±0.12; 2.75±0.14%) but was not significantly different ($p>0.05$) with B (2.89±0.17%). In addition, A showed the lowest Feed Conversion Ratio (FCR) was 1.25±0.07 which significantly different ($p<0.05$) with all the other treatments (Table 2).

In the end of the rearing period, there were the increasing of TVBC in the intestine of tilapia (Fig. 3a), in which the initial TVBC of all treatments were 8.43±0.19 log CFU g^{-1}. Treatment C showed the highest TVBC (13.20±0.12 log CFU g^{-1}) in the end of this study which was significantly different ($p<0.05$) with the other treatments. The improvement also occurred in TNP5 in the intestine of tilapia (Fig. 3b), in which there was no colony of *Bacillus* NP5 Rf[R] before the feeding trial. Treatment C showed the highest TNP5 (10.86±0.04 log CFU g^{-1}) in the end of this study which was significantly different ($p<0.05$) with the other treatments, in which TNP5 of A and B were 6.81±0.04; 8.79±0.02 log CFU g^{-1}, respectively while there was no colony of *Bacillus* NP5 Rf[R] in the control.

DISCUSSION

Drying is one of the methods for production of dried probiotic as the treatments in this study used. In addition, drying is also an encapsulation technique used for the active ingredient which dissolved in the coating material and forming an emulsion or suspension (Petrovic *et al*., 2007). Encapsulation is the process which a material or mixture of materials coated with or trapped in the material or other systems (Risch, 1995) which will produce particles with diameters varying from a few nanometers, micrometers to one millimeter (Anal and Singh, 2007; Zuidam and Shimoni, 2010). This technology is called microencapsulation and its products is called microencapsulated product. Microencapsulation through drying method which often used for laboratory and industrial scale are spray drying and freeze drying which are the physical methods of microencapsulation (Vidhyalakshmi *et al*., 2009; Bansode *et al*., 2010). Spray drying and freeze drying are widely used for the production of probiotics, because these methods are able to produce probiotics in powder form (Krasaekoopt *et al*., 2003) with stable quality. According to Zuidam and Shimoni (2010), spray drying can produce the final product as much as 5-50% while the percentage of microencapsulated product produced by freeze drying is so various. This was in line with the results of this study.

The coating materials used in drying method are the material which dissolved easily in the water like maltodextrin (Desai and Park, 2005; Zuidam and Shimoni, 2010). In addition, the materials also have an ability to protect the active compound during the process, able to form a film, dan cheap (Gharsallaoui *et al*., 2007). The higher probiotic viability of the treatments which using the coating material were caused by the coating material protected the probiotic from oxygen stress, heat and extreme environment during the drying process (Crittenden *et al*., 2006). This protection occurred because of

a film which formed by the coating material during the drying process (Reineccius, 2004). Otherwise, the lower viabilities of the uncoated treatments were caused by the extreme temperature during drying destroyed cell membrane, DNA and particular proteins within probiotic (Teixeira *et al*., 1997; Anal and Singh, 2007).

Besides a high product percentage and viability after drying, the probiotic product also must have a high viability in the storage period. In this current study, the viability of probiotic which dried by spray drying method was higher than freeze drying. This was in line with the previous study by Ying *et al*. (2010). Room temperature storage of microencapsulated probiotic which dried by spray drying method (WSRT) showed the higher viability because stability of the humidity of the product during storage, in which humidity will cause clotting or caking and then lead to the reduction of probiotic viability (Anekella, 2011).

In this study, the administration of dried *Bacillus* NP5 in feed by oral application was safe and able to improve the growth performance of tilapia, especially at a dose of 0.5% with concentration of 10^{10} CFU g^{-1}. Probiotic produces some digestive enzymes which can improve the feed utilization and the host digestion (Bairagi *et al*., 2002). *Bacillus* NP5 produces amylase (Putra, 2010) which plays a role in carbohydrate absorption. The dose of probiotic which given to the host have to be carefully determined to avoid the overdose that can give unexpected side effects and lost in production cost (Dash *et al*., 2014). In this study, the administration of probiotic in higher doses (1 and 2%) did not give a better result in the growth performance of tilapia. Probiotic in the very high dose will cause imbalance of the microbiota in the digestive tract and interfere immune response that can cause the lost of energy which used for the growth (Li *et al*., 2012; Ramos *et al*., 2013).

The enhancement of fish growth performance was also suggested caused by the presence of the intestinal microbial modulation that shown by the increasing of TVBC and TNP5 in the intestine of tilapia. This findings also similar with the previous study by Hoseinifar *et al*. (2011) who showed that the administration of probiotic could affect microbial community of the fish digestive tract by the increasing of LAB level in the digestive tract, in which LAB level could be the supporting factor of the growth performance improvement of beluga (*Huso huso*) juvenile. The TVBC of the treatments in this study were in the normal range as explained by Austin (2006) that bacterial population in the fish digestive tract is ~ 10^8 CFU g^{-1}. On the other hand, high metabolic activity of fish might be a cause for high bacterial load in the intestine as reported by Uddin and Al-Harbi (2012) who found TVBC in the intestine of carp and catfish ranged at $1.4\pm2.9\times10^{10}$ to $1.7\pm6.0\times10^{11}$ and $2.7\pm3.4\times10^{10}$ to $1.0\pm4.5\times10^{11}$ CFU g^{-1}, in which those numbers were higher than the normal range. In addition, the probiotic counts in this study were in line with Ziaei-Nejad *et al*. (2006) who revealed *Bacillus* numbers took 61.5-93.0% of total bacteria flora when Indian white shrimp larvae were inoculated with *Bacillus* via culture water or enriched Artemia.

CONCLUSION

Probiotic which encapsulated by maltodextrin and dried by spray drying method and stored in room temperature showed the higher product percentage, viability after drying and storage. This treatment was also able to maintain physical quality of the dried probiotic after being stored for a month. The administration of 0.5% dried *Bacillus* NP5 in feed by oral application showed the best growth performance in tilapia.

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