AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

Pathogenicity assay of probiotic-potential bacteria from the common carp *Cyprinus carpio*

[1]Cut Yulvizar, [2]Irma Dewiyanti, [1]Cut N. Defira, [2]Zainal A. Muchlisin, [1]Suhartono Suhartono

[1] Department of Biology, Faculty of Sciences, Syiah Kuala University, Banda Aceh 23111, Indonesia; [2] Department of Aquaculture, Faculty of Marine and Fisheries, Syiah Kuala University, Banda Aceh 23111, Indonesia. Corresponding author:C. Yulvizar, cut-yulvizar@unsyiah.ac.id

Abstract. The objective of the research was to determine the pathogenicity of probiotic-potential bacteria isolated from the common carp (*Cyprinus carpio*) collected from the Jantho region, Aceh Province, Indonesia. The research was conducted from July to September 2014 at Microbiology Laboratory and Hatchery Laboratory, Syiah Kuala University. A completely randomized design with single factor at five levels of treatment with three replications was used in this study. The treatment was A, positive control of *Aeromonas hydrophila*; B, the negative control of Phosphate Buffered Saline (PBS) solution; C, D, and E, probiotic-potential bacteria A, B, and C, respectively. The ANOVA test showed that the inoculation of probiotic-potential bacteria had a significant effect on the growth rate ($p < 0.05$), but did not have significant effect on the survival rate ($p > 0.05$). It was concluded that three isolates can be used as probiotic-potential bacteria based on pathogenity assay.
Key Words: common carp, pathogenicity, probiotic-potential bacteria, *Aeromonas hydrophila*.

Introduction. Common carp (*Cyprinus carpio*) is one of the most cultivated freshwater fish worldwide due to its considerable economic value (Stankovic et al 2011). There are various types and strains of common carps indicated in their physical forms, shapes and colors as a result of interaction between genotypes and habitats, seasons, and culture systems. This species was a favorite fish target for aquaculture farmers in Aceh Province, Indonesia especially in the Jantho region, Aceh Besar District. However, the farmers claimed that many cultivated carps were infected by diseases because of reduced production and profit. The farmers used antibiotics to control and inhibit the growth of pathogenic micro organisms; this drug was administered to treat bacterial infections. However, this method is costly and generates side effects not only for the bacterial pathogens but also for the cultivated fish. Prolonged use of antibiotics caused the pathogenic bacteria to be resistant and reduced the effectiveness of antibiotics. For the cultured fish, antibiotics may lead to bioaccumulation in the fish bodies creating a carcinogenic effect on humans who consume this contaminated fish (Bruno et al 2000).

The application of probiotic bacteria as biocontrol agents in fishery is one alternative to addressing the problems of diseases caused by bacterial pathogens (Irianto & Austin 2002). The incidence of microbial pathogens, especially those of bacterial origin, is one of the most significant factors affecting fish culture (Post 1989; Zorrilla et al 2003). The activity of probiotic bacteria can inhibit the growth of pathogenic bacteria without generating any adverse effects on the ecological balance system of bacteria (Feliatra et al 2004). Nowadays, use of probiotics in aquaculture is mostly focused on improving the digestion rate and growth performance of fish, while study on the probiotic potential bacteria for preventing disease is very limited. Therefore, the objective of the research was to analyze probiotic-potential bacteria which was effective to inhibit *Aeromonas hydrophila* growth and no pathogenic effect to common carp.

Material and Method. The research was conducted from July to September 2014 at Microbiology and Hatchery Laboratories, Syiah Kuala University.

Experimental design. A completely randomized design was applied in this study. Three isolates were obtained from the gut of the common carp based on *in vitro* selection criteria for probiotic-potential bacteria (Yulvizar et al 2014). Three types of probiotic-potential bacteria (A, B and C) and two controls (negative and positive controls) at three replicates were tested in this study, namely:
Treatment A = positive control: 0.1 mL $fish^{-1}$ of 10^6 CFU mL^{-1} of *Aeromonas hydrophila*;
Treatment B = negative control: phosphate-buffered saline (PBS) solution;
Treatment C = 0.1 mL $fish^{-1}$ of 10^6 CFU mL^{-1} of probiotic-potential bacteria A;
Treatment D = 0.1 mL $fish^{-1}$ of 10^6 CFU mL^{-1} of probiotic-potential bacteria B;
Treatment E = 0.1 mL $fish^{-1}$ of 10^6 CFU mL^{-1} of probiotic-potential bacteria C.

Pathogenicity assay of probiotic-potential bacteria. A total of 150 fish samples with sizes of 3-5 cm in length were collected from local farmers in the Jantho region. The fish were stored in 15 plastic containers (20 cm x 20 cm) with a stocking density of 10 fish per container and the fish were stored for one week for acclimatization. After acclimatizing, the fish were injectied by three different isolates of probiotic–potential bacteria in the lateral area of the body with a positive control of *A. hydrophila* and a negative control of.

The growth performance of the experimental fish was observed on a daily basis, while the survival rate was calculated at the end of the experiment. The results were compared with the positive control treatment of *A. hydrophila*, the negative control of PBS, and the injected probiotic-potential bacteria where the probiotic-potential bacteria selected in this study were non-pathogenic that do not show any symptoms of illness, stress or other abnormal conditions. Growth performance data were subjected to analysis of variance (ANOVA), followed by comparison of means using Duncan's multiple range test.

Results and Discussion. There were three probiotic-potential bacteria recovered from the stomachs and intestines of common carp where every bacterial isolate showed a variety of morphological features in both colonies and cells. Colonies of probiotic-potential bacteria isolates grown on Tryptone Soya Agar (TSA) had different forms, colors, sizes, and elevations. The colors of 3 probiotic potential bacterial were white, cream, grey, and yellowish white (Figure 1). Furthermore, the colonies showed difference in size and forms ranging from punctiform, circular, and irregular (Yulvizar et al 2014).

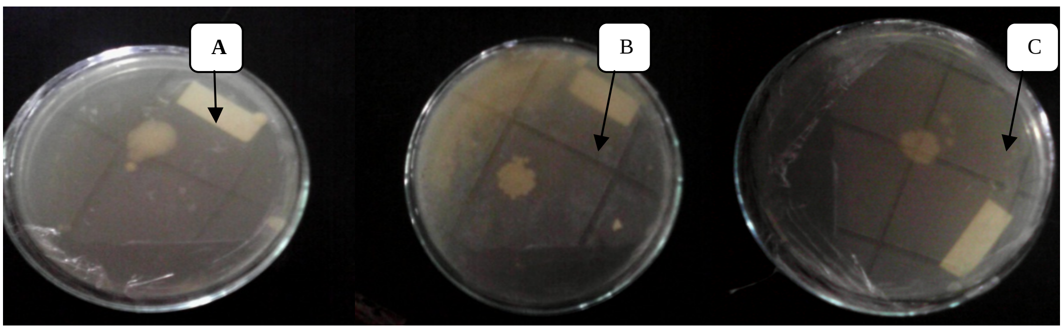

Figure 1. Isolates of probiotic-potential bacteria (isolat A; isolat B; isolat C).

The results of the pathogenicity assay of probiotic-potential bacteria are tabulated in Table 1. The ANOVA test showed that the isolate types had a significant effect on the growth rate ($p < 0.05$), but did not have a significant effect on the survival rate ($p > 0.05$). The study revealed that the growth rate of treated fish groups (treatment C, D and E) was significantly different from the control groups, which indicates that the probiotic-potential bacteria given to the fish had better results compared to the controls; therefore, these inoculates have the ability to inhibit and eliminate the pathogenic bacteria in aquaculture systems. There were not any clinical symptoms found in the fish treatment groups (B, C, D, and E). However, the fish in the control group had clinical symptoms after the injection of *A. hydrophila,* the positive control (treatment A), indicated by the alteration of morphology and behavior such as whirling and reduced feeding responses. In addition, morphological differences can be observed on hemorrhagic, ulcer, exophthalmia, and dropsy appearance in the fish of the positive control group. For example, the sample fish were bleeding at the injection site 18 hours post-infection (Figure 2A), and the fish showed poor appetite and gasping at the surface of the water after nine days post-infection. Exophthalmia occurred on the tenth day post-infection (Figure 2B) and the buildup of fluid inside the body cavity or tissues of the fish (dropsy) occurred on the twelfth day post-infection (Figure 2C).

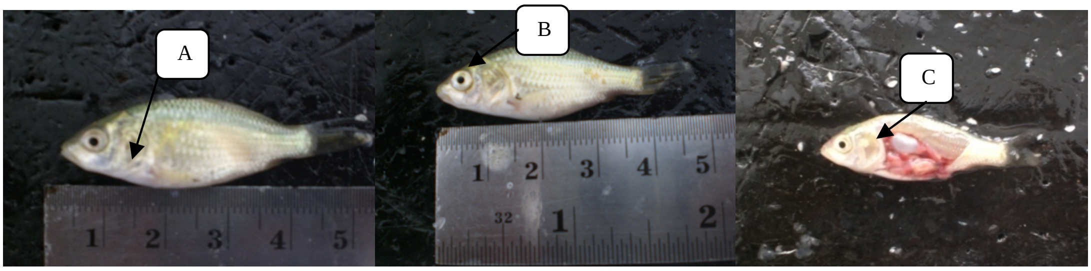

Figure 2. Clinical symptoms of indigenous carps: hemorrhagic (A); exophthalmia (B); and dropsy (C).

Table 1

Weight gain and survival rate of common carp (*C. carpio*) fry in vivo assay for 14 days. The mean values (±SD) in the same column with different superscrips were significantly different

Treatments	*Weight gain (g)*	*Survival rate (%)*
A	0.0149 ± 0.0067^{a}	73.33%
B	0.0963 ± 0.0068^{b}	100%
C	0.1146 ± 0.0189^{b}	100%
D	0.1157 ± 0.0099^{b}	100%
E	0.1036 ± 0.0042^{b}	100%

There were three bacterial isolates showing a variety of colony and cell morphology with white, cream, grey, and yellowish white colors as well as punctiform, circular, and irregular sizes and forms. This finding is similar to that of Agustina (2007) who reported that bacterial isolates from catfish (*Clarias* sp.) had transparent white, creamy white, creamy yellow and yellow colonies. A similar finding was also reported by Mulyati (2010) who isolated the bacteria from the intestines of some freshwater fish including java carp (*Barbonymus gonionotus*), catfish (*Clarias batrachus*), and tilapia (*Oreochromis niloticus*). A decrease in the survival rate of the experimental fish in the positive control was initially occurring from the ninth to the fourteenth day post infection during the assay. This might be due to an imbalance between bacterial activity and fish immune response. It was assumed that bacterial activity introduced by the *A. hydrophila* infection would be stronger and faster than the activity of the natural immunity of the fish. This speculation was supported by Denev et al (2009) who stated that a slower immune response cannot overcome the infection which may cause the fish stress and death. Another investigation using African catfish (*Clarias gariepinus*) infected with *A. hydrophila* found that the lowest survival rate of the positive control was 53.33% (Sukenda et al 2007). The most common clinical symptoms of the dead fish post *A. hydrophila* infection were ulcers, inflammation, hemorrhage, and necrosis. According to Bruno et al (2000) *A. hydrophila* excrete some toxic compounds leading to hemolytic. Therefore, hemolytic was the probable cause of fish death despite inflammation is the visible clinical abnormality observed in the fish.

The experimental fish exhibited morphological and behavioral alterations. The morphological alterations included bleeding on the skin surface (Figure 2A) at 18 hours post-infection followed by the appearance of ulcers. The bleeding that occurred on the skin surface might be due to hemolysis toxins. These toxins play an important role in the degradation of red blood cells so that the cells are out of blood vessels causing redness on the skin surface (Cipriano 2001; Huys et al 2002). The occurrence of ulcers might be due to the high density of bacteria in the injection area so that inflammation is severe at this section (Mangunwardoyo et al 2010). The *A. hydrophila* infections cause diseases with clinical symptoms such as loss of appetite, bleeding in the gills, enlarged abdomens filled with fluid, loose scales, fin tail off, swelling and tissue damage to the liver, kidneys and spleen (Post 1987; Austin & Austin 1999).

Direct observation showed that the fish lost their appetite and were gasping at the surface of the water nine days after the injection of A. *hydrophila*. Similar symptoms were also observed in some species of freshwater fish, inlcuding catfish (*Heteropneustes fossilis* and *Clarias batrachus*), carps (*Labeo rohita, Catla catla* and *Cirrhinus cirrhosus*) and perch (*Anabas testudineus*) (Sarkar & Rashid 2012) which indicates that one of the important characteristics of fish infected by *A. hydrophila* is reduced appetite. Another alteration to the fish was swelling of the eyes (exophthalmia), which corroborates the findings of Mangunwardoyo et al (2010) who reported that fish infected with *A. hydrophila* showed bleeding on the surface of the skin (hemorrhagic septicemia), bleeding at the base of the pectoral fin, muscle necrosis, ulcerative lesions (ulcer) on the surface of the body, and enlarged abdomens filled with fluid (dropsy). In addition, Asniatih et al (2013) stated that exophthalmia occurred as a result of an accumulation of fluids in the eye leading to concave and protruding eyes. Moreover, Austin & Austin (1999) stated that dropsy is a symptom characterized by the swelling of the fish body as a result of the release of cytotoxic Aerolysinenterotoxyn (ACT-gene) causing tissue damage.

The low weight gain in the positive control group might be due to the declining feed response of the fish after *A. hydrophila* infection. Plumb (1999) states that fish infected by motile aeromonad septicemia will lose their appetite. Kabata (1985) stated that reduced feed response is one of the symptoms of *A. hydrophila* infection. *A. hydrophila* infection can also cause bleeding in the liver (Sarkar & Rashid 2012) and a disruption of the liver affects the processes of metabolism (Cipriano 2001).

Conclusions. From these studies it was concluded that three isolates were isolated from the gut of the common carp based on in vitro selection criteria for probiotic-potential had a significant effect on the growth rate, but did not have a significant effect on the survival rate. Three isolates are safe and promising as probiotic-potential for cammon carp based on pathogenicity assay (in vivo selection).

Acknowledgements. This research was funded by the Directorate General of High Education (DGHE), Ministry of Education and Cultures, Republic of Indonesia through Syiah Kuala University, as a research grant for the 'Penelitian Hibah Bersaing' scheme in 2014. Therefore, the authors thank the DGHE Ministry of Education and Cultures, Republic of Indonesia and Syiah Kuala University for supporting this study.

References

Agustina, 2007 [Screening of probiotic bacteria to control *Aeromonas hydrophila* infection in catfish *Clarias* sp.]. Aquacultura Indonesiana 8(3):135-143 [in Indonesian].

Asniatih, Idris M., Sabilu K., 2013 [Pathological change of African catfish (*Clarias gariepinus*) infected by *Aeromonas hydrophila*]. Jurnal Mina Laut Indonesia 3(12):13-21 [in Indonesian].

Austin B., Austin D. A., 1999 Bacterial fish pathogens. In: Disease of farmed and wild fish. 3rd edition, Springer Praxis, Goldming, 457 pp.

Bruno G. G., Ana R., James F. T., 2000 The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191:259–270.

Cipriano R. C., 2001 *Aeromonas hydrophila* and motile aeromonad septicemia of fish. Fish diseases leaflet 68. United States Department of the Interior, Fish and Wild Life Service Division of Fisheries Research Washington DC, 25 pp.

Denev S., Staykov Y., Moutafchieva R., Beev G., 2009 Microbial ecology of the gastrosintestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. International Aquatic Research 1:1-29.

Feliatra I., Efendi, Suryadi E., 2004 Isolation and identification of probiotic bacteria from fish kerapu macan (*Epinephelus fuscogatus*) in fish feed efficiency efforts. J Nat Indonesia 6:75-80.

Huys G., Kampfer P., Albert M. J., Kuhn I., Denys R., Swings J., 2002 *Aeromonas hydrophila* subsp, dhakensis subsp. nov., isolated from children with diarrhoea in Bangladesh, and extended description of *Aeromonas hydrophila* subsp. *hydrophila* (Chester 1901) Stanier 1943 (approved lists 1980). International Journal of Systematic and Evolutionary Microbiologi 52:705-712.

Irianto A., Austin B., 2002 Probiotics in aquaculture. Journal of Fish Diseases 25:633-642.

Kabata Z., 1985 Parasites and diseases of fish cultured in the tropics. Taylor and Fancis Press, London and Philadelphia, 318 pp.

Mangunwardoyo W., Ismayasari R., Riani E., 2010 [Pathogenity and virulency assay to *Aeromonas hydrophila* in *Oreochromis niloticus* Lin, by Koch Postulat]. Jurnal Ristek Akuakultur 5(2):245-255 [in Indonesian].

Mulyati, 2010 [Screening of probiotic bacterium for controling Streptococcosis in tilapia *Oreochromis niloticus*]. Master Thesis, Institut Pertanian Bogor, Bogor, 112 pp. [in Indonesian]

Plumb J. A., 1999 Health maintenance and principal microbial diseases of cultured fishes. CRC Press, Tokyo, 149 pp.

Post G., 1987 Textbook of fish health. T. F. H. Publication, Inc. USA, 287 pp.

Sarkar M. J. A., Rashid M. M., 2012 Pathogenicity of bacterial isolate *Aeromonas hydrophila* to catfishes, carps and perch. Journal of the Bangladesh Agricultural University 10(1):157-161.

Stankovic M. B., Dulic Z. P., Markovic Z., 2011 Protein sources and their significance in carp (*Cyprinus carpio* L.) nutrition. Journal of Agricultural Sciences 56(1):75-86.

Sukenda Y. T., Trianggoro D., Wahyuningrum R., 2007 [Chitosan as control infection from *Vibrio harveyi* to udang putih *Litopenaeus vannamei*]. Jurnal Akuakultur Indonesia 6(2):205-209 [in Indonesian].

Yulvizar C., Irma D., Defira N. D., 2014 [In vitro antibacterial activity; selection potential probiotic from common carp (*Cyprinus carpio*) indigenous Jantho]. Teknologi dan Industri Pertanian Indonesia 6:2 [in Indonesian].

Zorrilla I., Chabrillon M., Arijo S., Diaz-Rosales P., Martinez-Manzanares E., Balebona M. C., Morinigo M. A., 2003 Bacteria recovered from diseased cultured gilhead sea bream (*Sparus aurata* L.) in southeastern Spain. Aquaculture 218:11-20.

Received: 8 July 2015. Accepted: 27 September 2015. Published online: 01 October 2015.

Authors:
Cut Yulvizar, Department of Biology, Faculty of Sciences, Syiah Kuala University, Banda Aceh 23111, Indonesia, e-mail: cut-yulvizar@unsyiah.ac.id
Irma Dewiyanti, Department of Aquaculture, Faculty of Marine and Fisheries, Syiah Kuala University, Banda Aceh 23111, Indonesia, e-mail: irma_alfian@yahoo.com
Cut N. Defira, Department of Biology, Faculty of Sciences, Syiah Kuala University, Banda Aceh 23111, Indonesia, e-mail: cutnandadevira@yahoo.com
Zainal Abidin Muchlisin, Department of Aquaculture, Faculty of Marine and Fisheries, Syiah Kuala University, Banda Aceh 23111, Indonesia, e-mail: muchlisinza@unsyiah.ac.id
Suhartono Suhartono, Department of Biology, Faculty of Sciences, Syiah Kuala University, Banda Aceh 23111, Indonesia, e-mail: suhartono1007@gmail.com

How to cite this article:
Yulvizar C., Dewiyanti I., Defira C. N., Muchlisin Z. A., Suhartono S., 2015 Pathogenicity assay of probiotic-potential bacteria from the common carp *Cyprinus carpio*. AACL Bioflux 8(5):694-698.