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THE CHARACTERISTICS AND VIABILITY OF PROTEOLYTIC BACTERIA CONTAINED WITHIN THE DIGESTIVE TRACT OF THE GROUPER FISH (*Epinephelus fuscogutatus*)

[1]Kasprijo, [2]Dulmiad Iriana, [2]Iskandar & [1]Tjahjo Winanto
[2]Faculty of Fisheries and Marine Sciences, Padjadjaran University, Bandung, Indonesia
[1]Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University, Purwokerto, Indonesia
Corresponding author's e-mail: kaspriyo37@yahoo.com

ABSTRACT
Proteolytic bacterium is one beneficial microbe which is potentially utilized to improve the microbe balance within the digestive tract. This research is conducted at the laboratory of the Faculty of Fisheries and Marine Sciences of Jenderal Soedirman University, Purwokerto. The purpose of this research is to obtain the proteolytic bacteria and examine their viability in the digestive tract of the grouper fish as the candidates of probiotic bacteria. This descriptive-qualitative research employs an explorative (discovery) strategy on the data obtained from the experimental results conducted at the laboratory. The research is conducted through observation and identification on the activities of proteolytic bacteria which are isolated from the digestive tract of the grouper fish (Barrow and Feltham, 1993). The research results show that there are four isolates of proteolytic bacteria. In addition, the identification and biochemical testing results show that those four bacteria are *Bacillus amyloliquefacient*, *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus licheniformis*. Thus, in the grouper fish farming, there are four potential isolates may be developed as the probiotics.

KEYWORDS: grouper fish, digestive tract, proteolytic bacteria, viability, and clear zone.

INTRODUCTION

Grouper fish is one export commodity with high economic value in the Asian markets, especially in Hongkong and Singapore. Grouper fish farming is continuously developed, mainly to improve the production. One alternative effort to improve the production is by accelerating the growth rate through the addition of probiotics on feed (Brilliant, 2014). Probiotics are products containing microbes which are beneficial to the hosts by accelerating the level of feed utilization and its nutrient value, improving the immune response of the hosts against various diseases or developing the surrounding environment (Verschuere *et al.*, 2000). The bacteria used as the probiotic candidates are the proteolytic bacteria (Beganovic *et al.*, 2013). Proteolytic bacteria are a group of bacteria with the capacity to break down proteins, by cutting the peptide bonds. There are various types of proteolytic bacteria, for examples, *Bacillus sp, E. coli, Klebsiella* and *Pseudomonas*. Those groups of bacteria may produce protease enzymes. In general, protease enzymes are divided into two groups covering proteinase (catalyzing the hydrolysis of protein molecules into peptides) and peptidase (hydrolyzing the peptide fragments into amino acids). Protease enzymes produced by the above proteolytic bacteria have various benefits. One benefit of protease enzymes is related to the nutrition increase (Suhartono, 1991). The application of feed containing the proteolytic bacteria which have probiotic characteristics may increase the efficiency of feed nutrient absorption within the digestive system. Thus, the application of probiotics may optimally accelerate the fish growth rate.

The research conducted by Ariole and Kanu (2014) states that proteolytic bacteria may be found in the fish digestive tract. There are microbes within the digestive tract which play an important role in the food digestion processes (Putra and Widanarni, 2015). The bacteria contained in the digestive tract show various enzyme potentials and play an important role in some functions of the host physiology (Bairagi *et al*., 2002). Based on that information, a research on the characteristics of proteolytic bacteria within the digestive tract of grouper fish (*E. fuscogutatus*) is conducted. The proteolytic bacteria selected from the digestive tract are expected to easily adapt to the digestive tract environment in order to assist the metabolism processes in the fish body.

MATERIALS AND METHODS

Material and Equipment

The materials and equipment used in this research are: grouper fish, NA liquid, NA solid, skim milk, distilled water, NaCl, $CaCo_3$, KCL, $MgSO_4$, crystal violet, iodine, alcohol 96%, sufranin, paper label and stationery, autoclave, microscope, Bunsen burner, scale, micro pipette, test tube, Erlenmeyer, Petri dish, object glass, inoculating loop, triangular spreader, magnetic stirrer, thermometer, incubator and refrigerator. All the equipment used should be first thoroughly washed, dried, and sterilized within the autoclave at the temperature of 121° C approximately for 15 minutes.

Research Method

Bacterial Sampling and Isolation

To obtain the digestive tract organ, first dissect the fish, wash thoroughly and put it into the physiological solution

of NaCl 0.9% at pH 2 (Feliatra *et al*., 2004). Isolate the probiotic bacteria from the digestive tract using a streak method, and then perform a series of dilution by taking 1 g sample, put it into a reaction tube containing 9 ml distilled water that the dilution 10^{-1} is eventually obtained. Meanwhile, to obtain the dilution 10^{-2}, take 1 ml of dilution 10^{-1}, put it into the reaction tube containing 9 ml distilled water, and continuously make a series of dilution to reach the dilution 10^{-5}. Take 1 ml of both dilution 10^{-4} and 10^{-5}, put it into a Petri dish containing NA medium, spread, and then incubate it in the inverted position for 24 hours at 30ºC (Darmayasa, 2008). Characterize the bacteria after the incubation for 24 hours and then isolate with a quadrant streak method for several stages that 1 pure isolate is obtained. Morphologically observe the obtained isolates, including the colony shape, edge, elevation and color (Barrow and Feltham, 1993). Meanwhile, the cell morphological observation includes gram staining and cell morphological test.

Cell morphology

Gram Staining

Clean the glass object with alcohol and pass it on the Bunsen burner flame several times, then aseptically take the bacterial isolate with the inoculating loop and smear it on the glass object. Drop the crystal violet staining on the bacterial isolate and let it stand for 1 minute, then wash it with the running water and leave it to dry. Drop the bacterial isolate once again with the iodine solution and let to stand for 1 minute, then wash it with the running water and leave it to dry. Furthermore, drop the bacterial isolate with alcohol 95% for 30 seconds, then flow it with the running water and leave it to dry. Drop the bacterial isolate with the safranin for 30 seconds and wash it with the running water, dry it with the absorbing paper and leave it to dry, and then observe it with the microscope. Gram-positive bacteria are characterized with the purple color indicating that the bacteria is able to bind the color of crystal violet, whereas gram-negative bacteria are characterized with the pink color indicating that the bacteria are unable to bind the crystal violet color and stained only with safranin (counter staining) (Hadioetomo 1993). Microscopically observe the growing bacteria's cell morphology on the preparation glass that the forms (coccus, stem, or spiral) may be shown, then morphologically and biochemically examined based on Barrow and Feltham (1993).

Enzyme activity Test

After being stored at the room temperature based on the treatment time, observe the single bacteria colony from the solid media using a pointed inoculating loop and grow it within the NA + skim milk media, then incubate it with the temperature of 35-37^{o}C. The protease activity of proteolytic bacteria is indicated by the presence of the clear zone around the colony (Susanti 2003).

Conduct a qualitative test to observe the clear zone shown by the bacterial colony, and then divide the clear zone's diameter with the colony's diameter. The results of the diameter division are relatively considered as the protease activity (Durham *et al.*, 1987). Proteolytic Index (IP) is the measuring equipment showing the ratio between the clear zone's diameter and the colony's diameter.

IP = Clear zone diameter/Colony diameter

The Determination of Bacterial Growth Phase

The accomplishment of bacteria's exponential phase may be determined by the bacterial growth phase. Make the culture preparation by inoculating 0.1ml of bacterial isolate into 10ml of liquid culture media and then incubate it for 24 h at the temperature of 29° C. This preparation is called fresh culture. Take 1 ml of the fresh culture and inoculate it into a 100 ml of sterile culture media and then incubate it once again at the temperature of 29°C. Subsequently, grow it within NA media; observe the bacterial growth every 4 hours. Barrow and Feltham (1993) mention that the development of *Bacillus sp* bacteria starts at 4th to 6th hours after incubation. The number of bacteria is calculated using the formula proposed by Madigan *et al*. (2003) as follows:

Total bacteria = number of colonies x 1/Fp x 1/S

Information:

Number of colonies = Number of colonies of the probiotic bacteria

Fp = Diluting factor (10^{-n})

S = Sample (ml)

Data analysis

The data resulted from the isolation and characterization of the proteolytic bacteria from the digestive tract of the grouper fish are analyzed using a descriptive method. The data are obtained from the results of proteolytic activity test while the proteolytic bacterial characterization is obtained from those of the biochemical test (Barrow and Feltham 1993).

RESULTS AND DISCUSSION

The Results of Bacterial Isolation from the digestive tract of the grouper fish

The research results show that the isolation of probiotic bacterial candidates from the digestive tract of the grouper fish grown within the NA media consists of four isolates which potentially become the probiotic bacteria. Those isolates have the colony morphology which is almost different. The observed colony morphology within the bacterial isolates includes the colony shape, edge, elevation and color (Figure-1). Based on the morphological characteristics, the bacterial genus of *Bacillus sp* is found in the digestive tract of the grouper fish with several morphological characteristics, such as the colony is in round shape, wavy edge, convex elevation and cream to white milk color. Hidayat *et al.* (2006) explains that the colony form of a bacterium is influenced by age and certain growth conditions. The existing bacterial form variations are also influenced by the environment (biotic and abiotic factors), food, and temperature (Ilyas, 2001).

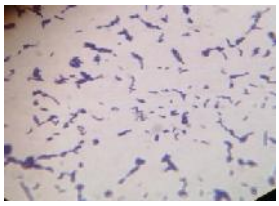
A  B  C 

FIGURE 1. **A**. Bacterial Colony **B.** Bacterial Colony Purification **C**. Positive Gram

TABLE 1. Colony and Cell Morphology as well as the bacterial isolate biochemistry and physiology obtained from the digestive tract of the grouper fish

Uji Bio Kimia	Kode Isolat bakteri			
	GR - 5	GR - 6	AL-2	AL-4
Shape	R	R	R	R
Gram	+	+	+	+
Motility	+	+	+	+
Cell long >3 µm	–	–	–	–
Position and spora shape	VX	VX	VX	VX
Spora	+	+	+	+
Growth on 50^{o}	+	+	–	+
Growth on 37^{o}C	+	+	+	+
Growth with 10% NaCl:	+	+	+	–
An aerobik	–	–	+^{F} (W)	+^{F}
Aerobik	–	–	–	–
Acid from phenol red (glukose)	+	+	+	+
OF (glukose)	–	–	–	–
Acid from ASS medium				
- Glucose	+	+	+	+
- Celibiose	+	+	+	+
- Galactose	–	–	–	–
- Mannose	+	+	+	+
- Melibiose	–	+	+	+
- Rafinose	+	+	+	+
- Salicin	–	–	–	+
- Xylose	–	+	+	+
ONPG	+	+	+	+
Utilization of Citrat	–	+	+	+
Urease	–	–	–	–
Indol	–	–	–	–
VP	+	+	+	+
Nitrate reduced	+	+	+	+
Casein hydrolysis	+	+	+	+
Starch hydrolysis	+	+	+	–
Oksidase	+	+	+	+
Katalase	+	+	+	+

VX: Central/oval, R: Road-shape, +^{F}**:** Fakultatif an aerobic, GR-5: *Bacillus amyloliquefacient*, GR-6: *Bacillus subtilis*, AL-2: *Bacillus pumilus*, AL- 4: *Bacillus licheniformis*

The colony color which differently appears shows that there are pigment differences. Savitri (2006) describes that the pigments contained in bacteria include carotenoid, anthocyanin, melanin, Tripirilmethene, and Phenazin, in which each pigment may give a different color. Holt *et al.* (1994) suggest that *Bacillus sp.* bacteria belong to the bacterial colonies with white milk-to-yellowish color with wavy edge, widespread in various habitats with few pathogenic species. Feliatra *et al.* (2004) explain that the bacterial genus of Bacillus sp are found in the digestive tract organ of the grouper fish with some morphological characteristics, such as the colony is in white color with a round shape and wrinkle edge.

To figure out whether or not *Bacillus sp* is included into probiotic bacteria, then gram staining test is conducted. The testing results show that *Bacillus sp* is categorized into gram positive bacteria with the results of gram staining test of purple (Figure 1). Gram-positive bacteria through the gram staining test has the color characteristic which is looked purple due to the ribonucleic acids in the cytoplasm form a stronger crystal violet bond that the chemical bond is not easily solved by the color lightener (Hadioetomo, 1993). Feliatra *et al.* (2004) describe that one main indicator of probiotic bacteria is included into positive gram bacteria and not pathogenic.

Based on the results of phenotipic observation and biochemical tests, it is found that there are four species of Bacillus sp genus which characteristics and cell morphology are completely different (Barrow and Feltham 1993). (Table. 1) Those bacteria are *B. amyloliquefacient, B. Subtilis, B. Pumilus, and B. licheniformis.*

Mustaqim *et al*, (2014) explains that the bacterial population of *B. subtilis* and *B. licheniformis* is found along the digestive tract of the grouper fish with the

species number and existence highly influenced by the type and age of the fish species – the bacterial species may be found in those fish living in the fresh water and sea water environments (Moriarty 1999). Barrow and Feltham (1993) mentions that *B. pumilus* and *B. licheniformis* are positive facultative anaerobic bacteria while *B. amyloliquefacient* and *B. subtilis* are negative aerobic bacteria with rod-shaped cells, classified into gram-positive bacteria in the young culture, motile (non motile reaction occasionally occurs), producing spores which are usually resistant to heat, positive catalase, and positive oxidation.

Proteolytic Activity Test

The results of proteolytic activity test are characterized by the clear zone existence around the colony isolate. The clear zone indicates that the bacteria have been able to hydrolyze the casein contained in the skim milk agar media. The diameter measurement results of the proteolytic activity test at the 0 to 8 hours storage time of protease enzyme activity are not yet clearly seen as the clear zone is not yet formed. At the12-hours storage time of enzyme activity, the clear zone formation starts to be seen with the size of about 1.2 to 1.3 cm. The development of the highest proteolytic enzyme activity is produced by *B. amyloliquefacien* bacteria with the protease activity index value reaching 1.8 at the 24-hours storage time followed by *B. subtilis, B. Pumilus, and B.licheniformis* with the proteolytic activity index respectively by 1.6, 1.5, and 1.4. It is assumed that B. *Amyloquefacien* bacteria have higher protease enzyme activity when compared to the other three species. Barrow and Feltham (1993) explain that each bacterium's enzyme activity is different depending on its species. The enzyme activity improvement may be caused by the presence of substrates within the media. Dewi (2004) suggests that the presence of substrate within the production media may trigger bacteria to secrete their cell metabolites that the enzyme activity and productivity may also be influenced. If the substrate exists within a medium, the enzyme may work to form a product that its enzyme activity will be high. Conversely, if the substrate in a medium is reduced, then the enzyme may not optimally work in forming a product that its enzyme activity will be low. Thus, the longer the storage time, the lower substrate may become that the enzyme activity and productivity may also decrease. The other factors may occur as Mavitra (2005) explains that the decreasing enzyme activity is due to the increasing amino acid content in the medium. This amino acid accumulation acts as a co-receptor which is bound to the receptor that the receptor is able to bind the operator and block the synthesis processes. Pelezar and Chan (1986) mention the factors which influence the enzyme activity are the enzyme concentration, substrate concentration, pH, and temperature. The higher the enzyme concentration the higher the enzyme activity will be. Ph also influences the enzyme activity, in which the maximum activity is achieved at a given pH. Temperature also influences the enzyme activity which increases due to the rising temperature that optimum activity may be reached. The extreme temperature increase eventually results in the reduced enzyme activity that the destruction of enzyme eventually occurs.

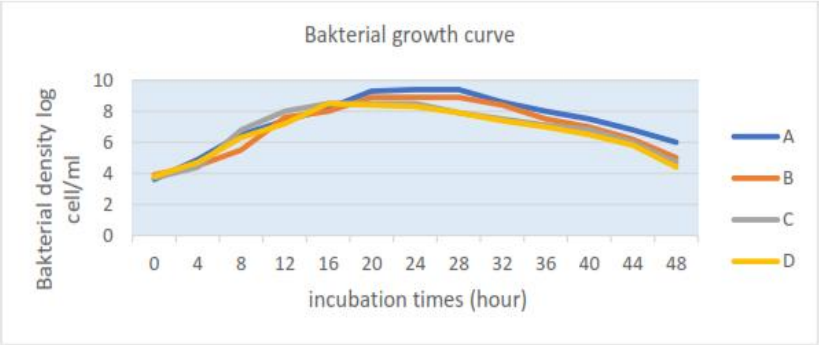
During the growth, the proteolytic bacteria may produce protease enzyme. If the enzyme is combined with the substrate, it may become substrate complex, which then breaks down into product (Pelezar and Chan, 1986). Protease enzyme breaks down the complex compounds into simpler compounds that those may be absorbed by the cells and utilized for growth. The enzyme activity in breaking down the complex compounds into simpler compounds may be figured out due to the presence of clear zone around the bacterial colony. The increased enzyme activity is proven with the clear zone's increasing diameter around the bacterial colony resulted from the hydrolysis with skim milk media which is used as the protein source for the bacterial growth.

A qualitative hydrolysis activity is the description of proteolytic bacteria's ability to form a clear zone around the isolates grown in the skim milk agar media. The qualitative testing media in this research uses milk agar media. Hapsari (2016) states that milk is a perfect medium for the bacterial growth due to its various nutrient contents. Casein is a milk protein consisting of phosphoprotein bound to the calcium to form a calcium salt called calcium calcinate. This molecule is very large and does not dissolve in the water and also form colloids. This suspension has a white color and may be directly observed when suspended in a solid culture media. The clear zone formed around the bacterial colony is the sign of casein particle loss in the skim milk media. Due to the presence of proteolytic enzymes of extracellular bacteria, the casein will be hydrolyzed into peptide and soluble amino acids. The extracellular enzyme of *Bacillus sp* is very efficient in breaking down various carbohydrate, lipid and long chain protein compounds into short chain units or simpler compounds.

The Viability of Proteolytic Bacteria

After being stored in the incubator for 24 hours, the bacterial colony growing within NA medium may be then calculated. The *B. amyloliquefacient*, *B. Subtilis*, *B. pumilus*, and *B. licheniformis* bacteria have the external colony morphology which is almost the same with the white color, round shape, the wavy edge. The bacterial colony counting is made during the 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 hours storage. Graph1 shows that the growth of those four species' increase starts from the 4th hour observation. The logarithmic phase starts to occur from 0 to 16 hours storage, with the maximum bacterial growth of *B. pumilus*, and *B. licheniformis*, which each is $3.3x10^8$ and $2.9x10^8$ cells /ml while the logarithm phase of *B. subtilis* and *B. Amyloliquefacient* starts to occur from 0 to 20 hours, which each is 2.9×10^9 and $7.6x10^8$ cells/ml. The logarithmic phase is a phase when the bacteria are able to maximally develop and the numbers follow the logarithmic curve that the perfect incubation period is known before the harvest time (Fardiaz 1992). The 16^{th} hour is the optimum storage time for *B. pumilus*, and *B. licheniformis* bacteria while the 20^{th} hour is the optimum storage time for *B. amyloliquefacient* and B. *subtilis* bacteria. The bacterial optimum growth is due to the substrate within the media which is still able to provide nutrients or to support the life of the growing bacterial population. Naughton and Larry (1990) describe that the peak population number is positively related to the

food/nutrition intake. The optimum growth is also due to the presence of high metabolic activity in synthesizing the contained substances and the division maximum speed. Mavitra (2005) explains during the growth period, the cells' metabolism activity and division speed are high, while the generation period is short. This quite high increase occurs since the bacteria synthesize the substances contained within the media which may trigger the bacteria in expelling the cell metabolite for the optimum cell growth. High protein content in the medium may be utilized as the nutrient sources for the proteolytic bacteria.



GRAPH 1: Bacterial growth curve of A. *B. amyloliquefacient*, B. *B. subtilis*, C. *B. pumilus*, and D. *B. licheniformis*

On the probiotic bacterial growth curve, there is no adaptation or lag phase since the culture used in the growth curve measurement is the culture (preculture) grown before the measurement with the same medium that reducing the adaptation time. The stationer phase of *B. pumilus* and *B. licheniformis* bacteria start to occur to occur at the 16th to the 24th hour, while the stationary phase of *B. subtilis* and *B. Amyloliquefacient* bacteria start to occur to occur at the 20th to the 28th hour when entering this phase, the biomass concentration is maximum, the number of cells tends to be stable, the growth stops and the modification of cell biochemical structure occurs. The bacterial growth rate of *B. pumilus* and *B. licheniformis* decline, while *B. subtilis* and B. *amyloliquefacient* starts to occur at the 24th, 28th, 32nd and 36th after the 2nd incubation period. The decline occurs due to the lessened growth factors, such as the declining supplies of the contained nutrients. Naughton and Larry (1990) state that the organism growth and survival are lessen due to the limited supply of food/nutrients. Under the limited food conditions, then the number of organisms may rapidly decline. In addition, the growth rate decreases due to the increasing mortality, decreasing reproduction or suspected that the probiotic bacteria experience many deaths caused by several factors, such as the availability of nutrients in the media is reduced, the reserved energy within the cells runs out, the accumulation of acid and the other metabolites.

Widyastuti (1995) states that the factor influencing the bacterial viability decrease is the length of the storage time. Gaman and Sherrington (1992) also describe that the factors influencing the bacterial growth in general include time, nutrition, water, temperature, oxygen, and pH. All bacteria require nutrients to grow. If the nutrients are inadequate, the growth may decrease. Water is required by the bacteria as water plays an important role in the cells' metabolic reactions. Highly concentrated Media may cause the cells experience deficiency of water and then die. Temperature is one most important environmental factor which influences the bacterial growth. If the temperature rises, the metabolic rate increases, the growth is then accelerated, and vice versa. The influence of oxygen consumption depends on the types of bacteria (aerobic or anaerobic). In addition, bacteria grow very well at pH 6.6 and 7.5 (neutral).

CONCLUSION

The isolation results of proteolytic bacteria from the digestive tract of the grouper fish (*E. fuscogutatus*) show that there are four bacterial species which have specific characteristics, namely *B. amyloliquefacient*, *B. Subtilis*, *B. Pumilus* and *B.licheniformis*. The proteolytic index value of 1.8 1.6, 1.5, and 1.4, respectively. The bacterial logarithmic phases of *B. Pumilus*, and *B. licheniformis* start to occur from the storing time of 0 to 16, with maximum growth of $3.3x10^8$ and $2.9x10^8$ cells/ml while the bacterial logarithmic phase of *B. amyloliquefacient* and *B. subtilis* start to occur from 0 to 20 hours of storage with the maximum growth of $2.9x10^9$ and $7.6x10^8$ cells/ml respectively.

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