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ISOLATION, CHARACTERIZATION, AND IDENTIFICATION OF PROTEOLYTIC BACTERIA TO IMPROVE PROTEIN DIGESTIBILITY OF FISH FEED INGREDIENTS

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ABSTRACT

Protease is one of the potential enzymes used to improve the quality of local raw materials as fish feedstuff. The aim of this research was to find high protease activity-bacteria isolates in improving protein digestibility of fish feedstuff. Isolation was conducted on various growth substrates such as water and sediment of hot springs, mangrove sediment, and shrimp head waste. The parameters used in the selection and characterization of potential proteolytic bacteria were qualitative and quantitative tests of proteolytic activity. Microbes that have the highest enzyme activity were then selected and characterized based on their growth curve, production time of protease, and determination of optimum condition (temperature and pH) of protease activity. The result showed that there are only 18 isolates from 44 isolates positively had protease activity (qualitatively). Three isolates with the highest activity (RGL1.1, PL1.1, and PL1.2) qualitatively and quantitatively were then selected to be tested for their pathogenecity on tilapia. The results showed that only RGL1.1 was not pathogenic and therefore, potentially used as a protease producer candidate. The characterization of RGL1.1 isolates showed that the exponential phase of RGL1.1 was thirty-sixth hour in line with the optimum activity of enzyme production. The optimum pH and temperature of RGL1.1 isolate were 6 and 50°C, respectively. RGL1.1 isolate is a Gram-positive rod-shaped bacteria that has 99.53% of resemblance to *Bacillus cereus*.

KEYWORDS: proteolytic bacteria; isolation; characterization; identification

INTRODUCTION

Protease is one of the important enzymes for industrial and agricultural biotechnology applications. This enzyme has been used extensively in animal feed industry and contributed to 65% of the total sales of enzymes in the world (Badriyah & Ardyati, 2013). Protease (E.C.3.4) is a hydrolase class enzyme that plays a role in the peptide-binding reaction to protein molecules. This enzyme catalyzes the hydrolysis reaction, a reaction involving the water element to the specific bond of the substrate (Kurniawan, 2015).

Similar to the animal feed industry, protease can potentially be used to improve the quality of fish feed ingredients in aquaculture. This is because the proportion of indigestible protein in the average raw material is still relatively high. For example, the undigested protein in sorghum, wheat, corn, soybean, and wheat bran or rice bran are 22%, 23%, 27.3%, 20.7%, and 28.8%, respectively (Marlina, 2014). In addition, some anti-nutritional factors in the protein of feed ingredients such as lectin and trypsin inhibitors can trigger damages to the absorption surface, due to the imperfection of the digestive process. The addition of proteases can help neutralize the negative effects of these protein factors and also break down hard-to-digest proteins into simpler, more readily absorbed molecules. Protease can also be used to improve the quality of several potential feed ingredients such as blood meal, poultry meal, and shrimp head or shrimp skin meal.

Protease can be produced by microorganisms such as bacteria. The use of microorganisms as sources of enzymes has several advantages over enzymes isolated from plants or animals. Microorganism cells are relatively more easily to be grown, have relatively faster growth rates, can easily be produced in large quantities when needed, and are cost effective. The production of microorganism cell is not dependent on seasonal changes, need shorter production time (Rahman & Indarto, 2013). Such advantages mean that

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it is important to explore, isolate, characterize, and identify potential protease-producing bacteria in order to improve protein digestibility of fish feed ingredients. Protease-producing bacteria can be isolated from various growth substrates such as hot springs, mangroves, shrimp wastes, and others. The aim of this study was to obtain bacterial isolates that have high protease activity and potentially be used to improve protein digestibility of fish feedstuff.

MATERIALS AND METHODS

The analysis was conducted at the Nutrition and Feed Technology Laboratory, Research Center and Development of Freshwater Aquaculture. Sampling were conducted in Saguling, Cianjur; Ciseeng, Bogor; White crater; and Rengganis crater in Bandung; Eretan, Indramayu; Muara Angke, Jakarta from February to October 2016.

Exploration and Isolation of Proteolytic Bacteria from Various Growth Substrates

The first stage of this research was the exploration and isolation of microbes on various growth substrates such as hot spring water and sediment, mangrove sediment, and shrimp head. The samples were diluted gradually and 100 µL of samples were collected and dripped onto a skimmed milk medium agar, flattened and incubated at 37°C for ± 48 hours. Each colony was purified during the growing period. After 48 hours, the growing colony then was observed macroscopically to determine its shape, size, texture, and color.

Selection and Characterization of Protease-Producing Bacteria from Various Growth Substrates

The next stage of this research was the selection and characterization of proteolytic bacteria. Several tests were conducted in the selection of proteolytic bacteria including qualitative and quantitative tests of proteolytic activity as well as pathogenicity test. Isolate that has the highest enzyme activity and not pathogenic to fish was then selected as the candidate of proteolytic bacteria. After that, the isolate was characterized by its microbial growth curve, production time of protease enzyme, and protease enzyme activity optimum conditions (temperature and optimum pH).

Selection of proteolytic bacteria

Qualitative test of proteolytic activity

Qualitative proteolytic test of the isolates was done by using skim milk agar (SMA) media. Each isolate was placed into a petri dish containing SMA media and then incubated for 24 hours at 37°C. Proteolytic activity is indicated by the appearance of clear zones around the formed colonies. The calculation of proteolytic index is the ratio of total zone diameter extending from the center of the bacterial colonies. The proteolytic index is calculated as follows (Firliani *et al*., 2015):

$$\text{Proteolytic index} = \frac{(a-b)}{b}$$

where:
a = the diameter of the total zone
b = diameter of colony

Quantitative proteolytic activity test

1 mL of the isolate was cultured in 9 mL Trypticase Soy Broth (TSB) containing skim milk and incubated at 28°C for 48 hours. The culture was then centrifuged at 5,000 rpm for 15 min. at 4°C. The supernatant obtained was a crude enzyme extract ready to use in the proteolytic activity test.

Proteolytic activity is determined based on the method of Bergmeyer & Grassi (1983) (Table 1). Casein was hydrolyzed by proteases into peptides and amino acids. The amino acids were separated from the remaining concentrate by the addition of trichloroacetic acid (TCA) or perchloric acid. The amino acids formed were dissolved in TCA, whereas the non-hydrolyzed protein was settled in the presence of TCA. The isolated amino acids were measured their absorbance at a 280 nm wavelength or colored first with a folin-ciocalteau reagent at a wavelength of 650 nm. One unit of activity represents the amount of enzyme that can produce one micromole of tyrosine per minute (Bergmeyer & Grassi, 1983).

The activity of the protease is calculated by the formula:

$$\frac{U}{mL} = \left[\frac{ODs - ODb}{ODstd - ODb}\right] DF \times \frac{1}{T}$$

where:
$\frac{U}{mL}$ = activities in internatio nal units per minute
ODs = absorbance of samples
ODb = absorbance of blanks
Odstd = absorbance of standards
DF = dilution factor
T = incubation time

Characterization of proteolytic bacteria in producing protease enzymes

Pathogenicity test

The test was performed to determine the pathogenicity of proteolytic bacteria. All chosen isolates

Table 1. Protease activity test

Ingredients*	Blank (mL)	Standard (mL)	Sample (mL)
Buffer phosphate (0.05 M; pH 8.0)	1.00	1.00	1.00
Casein (20 mg/mL; pH 8.0)	1.00	1.00	1.00
Enzyme on $CaCl_2$ (2 mmol/L)	-	-	0.2
Tyrosine standard (5 mmol/L)	-	0.20	-
Aquadest	0.20	-	-
Incubated at 37°C for 10 minute			
TCA (0.1 M)	2.00	2.00	2.00
Aquadest	-	-	0.20
Enzymeon $CaCl_2$ (2 mmol/L)	0.20	0.20	-
Incubated at 37°C for 10 minute, then centrifuge at 3,500 rpm for 10 minute			
Filtrat	1.50	1.50	1.50
Na_2CO_3 (0.4 M)	5.00	5.00	5.00
Folin ciocalteau	1.00	1.00	1.00

(RGL1.1, PL1.1, and PL2.1) were cultured for 24 hours at 28°C. Each isolate, with a concentration of 10^8 cfu/mL and at a dose of 1 mL, were then injected intramuscularly and intraperitoneally into the body of five healthy tilapia measuring an average weight of 10 g. Another group of healthy tilapia (five individuals) was injected with phosphate buffer solution of pH 7 with a dose of 1 mL as a control. Fish mortality, sickness, and abnormal activity were observed for two weeks. Each fish group used in the pathogenicity test was kept separately in two 60 cm x 50 cm x 40 cm aquarium.

Determination of growth curve of proteolytic bacteria and optimum protease production time

Pure rejuvenated isolates were cultivated in a fermented/production medium to study the protease activity. This process began with the production of inoculum then followed by the fermentation process (enzyme production). The inoculum, which has been incubated for 18-24 hours at 30°C, was taken as much as 10% to be inoculated into 100 mL of production medium. In every six hours, a sampling was performed to measure bacterial growth known as the optical density (OD) measurement at 650 nm wavelength. The measurement of protease enzyme activity used enzymatic method (Bergmeyer & Grassi, 1983).

Determination of optimum temperature and pH for proteolytic activity

The optimum temperature and pH for proteolytic activity were determined as follow: the bacteria were cultured in a solution containing skim milk broth then incubated for 48 hours at temperature ranges of 30°C, 40°C, 50°C, and 60°C and at pH ranges of 5, 6, 7, and 8. Afterward, the bacteria were tested for their proteolytic activity at the temperature and pH ranges until the optimum proteolytic activity of certain temperatures and pH were observed.

Identification of Selected Proteolytic Bacteria

The final stage of this study was the identification of the selected isolates through a series of morphological, biochemical, and molecular tests. The isolates were morphologically characterized their gram staining response, spore, and motility. The biochemical tests consisted of catalase, peptone, and gelatin. Molecular identification was conducted through bacterial DNA isolation performed using the PCR method (Packeiser *et al*., 2013). Cells from a single colony on a solid surface media were taken using sterile toothpicks and suspended into 50 µL of nuclease-free water. Cell lysis was performed with a vortex suspension for 10 seconds and incubated at 98°C for five minutes. The lysate was further spun down to separate the supernatant from the cell debris. The supernatant was taken and used as a DNA template in PCR amplification.

The amplification of 16S rDNA fragments was performed using GoTaq (Promega) with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Zhang *et al*., 2009; Palaniappan *et al*., 2010). The purification of PCR products was performed following the PEG precipitation method (Hiraishi *et al*., 1995) and continued with se-

quencing cycles. The results of the sequencing cycles were regrouped using the ethanol purification method. The order of nitrogen bases was analyzed using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The sequenced data were then processed with the Bioedit program. The homology of 16S rDNA sequence was searched in Eztaxon servers online database (Kim *et al*., 2012).

RESULTS AND DISCUSSION

Exploration and Isolation of Proteolytic Bacteria

Protease-producing bacteria were selected and isolated from hot spring, crater, and mangrove sediments as well as shrimp head. The bacteria isolated from various growth substrates are presented in Table 2.

Selection and Characterization of Protease-Producing Bacteria

The results revealed that only 18 isolates of bacteria had proteolytic activity characterized by the formation of clear zones on agar media (Table 1). Nine isolates that had the highest proteolytic index were selected. The isolate that had the highest proteolytic index was obtained from Parung hot water sludge coded as PL1.2 (Table 3). Six of nine bacteria isolates with the highest proteolytic index had high protease activity of which, the highest activity was produced by bacterial isolate RGL1.1 (Table 4).

Characterization of proteolytic bacteria in producing protease enzymes

Pathogenicity test

Three isolates that had the highest enzyme activity were RGL1.1, PL1.1, and PL2.1. These isolates were subjected to the pathogenicity test to determine if they were a pathogen or not. The results showed that tilapia injected with PL1.1 and PL2.1 had died within 24 hours after injection. Seven fish injected with RGL1.1 had still alive during the pathogenicity tests while three other had died due to injuries caused by the injections. This indicates that RGL1.1 was not pathogenic to tilapia and can potentially be used as a candidate of protease producing bacteria to improve the quality of fish feedstuff.

Growth curve of proteolytic bacteria and optimum protease production time

Based on the growth curve, the lag phase of RGL1.1 isolate is from the initial time to the sixth hour of incubation time (Figure 1). The lag phase or adaptation phase is a condition in which nutritious microbial cells in previous cultures begin to adapt to their new environment (Morse & Mietzner, 2013). The exponential phase of this isolate was quite long from the sixth hour until the thirty-sixth hour of incubation time. In the exponential phase, bacterial cells divide and cell metabolism progresses rapidly resulting in the increasing number of cells that keep going continuously until reaching a stationary phase (Sunaryanto, 2011). The stationary phase of RGL1.1. isolate began after the thirty-sixth hour marked by a decreased in bacterial growth until the forty-eighth hour. In this phase, the rate of bacterial growth was proportional to the rate of cell death and the accumulation of toxic metabolites in culture media. In this phase, the bacteria began to produce enzymes and other metabolites. This is due to the competition between cells over the increasingly depleted nutrients (Morse & Mietzner, 2013).

Based on the protease production curve presented in Figure 2, it can be concluded that protease activity correlates positively to bacterial growth. The greater the number of cells, the greater the protease activity produced. The results showed that at the 36th hour, the bacteria produced the highest protease activity, which was 0.089 U/mL and indicated the optimum incubation time.

pH and Temperature Optimum

The effect of pH on protease enzyme activity is presented in Figure 3. This study found that protease activity of bacteria isolated from hot spring sediment of Rengganis crater has an optimum condition at pH 6 considering that the optimum pH range of protease production varies according to its protease type. However, the optimum pH of protease in this study was still within the pH range between acidic and neutral. This is in line with Suhartono (1989), who reported that bacteria such as *Bacillus subtilis* could produce three kinds of protease enzyme namely acid, neutral, and alkaline proteases. At an optimum pH, the enzyme has an active side conformation that matches to the casein substrate leading to a maximum formation of a substrate-enzyme complex. At this point, the giver and receiver proton groups on the catalytic side of the enzyme are at the desired ionization level, which will generate the maximum product (Suhartono, 1989).

The catalytic activity of protease enzyme is also affected by temperature. Low temperature slows down the chemical reaction and higher temperature causes the reaction to occur more rapidly. The effect of temperature variations on RGL1.1 protease activity is presented in Figure 4. The protease activity of RGL1.1

Table 2. Isolated proteolytic bacteria from various growth substrates coded according to the source locations

Location		Isolate code	Proteolytic activity
Saguling, Cianjur	- Hot spring	SGA1	-
		SGA2	+
	- Sediment/sluge	SGL1	-
		SGL2	+
White crater, Bandung	- Hot spring	-	
	- Sediment/sluge	CW2L1	-
		CW2L2	-
Rengganis crater, Bandung	- Hot spring	RGA3.1	-
		RGA3.2	-
	- Sediment/sluge	RGL1.1	+
		RGL1.2	+
		RGL1.3	-
		RGL2.1	-
		RGL2.2	+
		RGL3.1	+
		RGL3.2	-
Ciseeng, Parung, Bogor	- Hot spring	PA1	+
		PA2	-
		PA3	-
	- Sediment/sluge	PL1.1	+
		PL1.2	+
		PL2.1	+
		PL2.2	+
		PL3.1	-
		PL4.1	+
		PL4.2	+
Mangrove and catfish pond; Eretan, Indramayu	- Mangrove sediment	IM1L1	-
		IM1L2	-
		IM1L3	-
		IM2L1	+
		IM2L2	-
		IM2L3	+
	- Mangrove water	IM1A1	-
	- Pond water	IM3A1	-
		IM3A2	-
	- Pond sediment	IM3L1	-
		IM3L2	-
Muara Angke, Jakarta	- Shrimp head waste	MA1.1	-
		MA1.2	-
		MA1.3	-
		MA2.1	+
		MA2.2	-
		MA2.3	+
		MA2.4	+

Table 3. Proteolytic index of bacteria isolated from different growth substrates

Isolates code	Colony diameter (mm)	Total zone (mm)	Proteolytic index
RGL.1.1	6	16	1.7
RGL1.2	5	15	2
PL.1.1	6	9	0.5
PL1.2	4	17	3.3
PL2.1	6	15	1.5
PL2.2	5	10	1
MA2.1	7	9	0.3
IM2L3	3	9	2
IM2.L1	2	7	2.5

Table 4. Protease activity of isolates from various growth substrates measured quantitatively

Isolate code	Proteolytic activity (U/mL)
IM2L1.1	0.007 ± 0.001
PL1.1	0.023 ± 0.001
RGL1.1	0.100 ± 0.006
PL2.1	0.015 ± 0.004
PL2.2	0.0023 ± 0.0004
RG1.2	0.004 ± 0.003

Table 5. Pathogenicity test of proteolytic bacteria on tilapia (*Oreochromis niloticus*) for two weeks

Isolate code	Phatogenicity (two weeks)	
	Alive fish (ind.)	Dead fish (ind.)
RGL1.1	7	3
PL1.1	0	10*
PL2.1	0	10*

Notes: * Fish died after 24-hour post injection of proteolytic bacteria

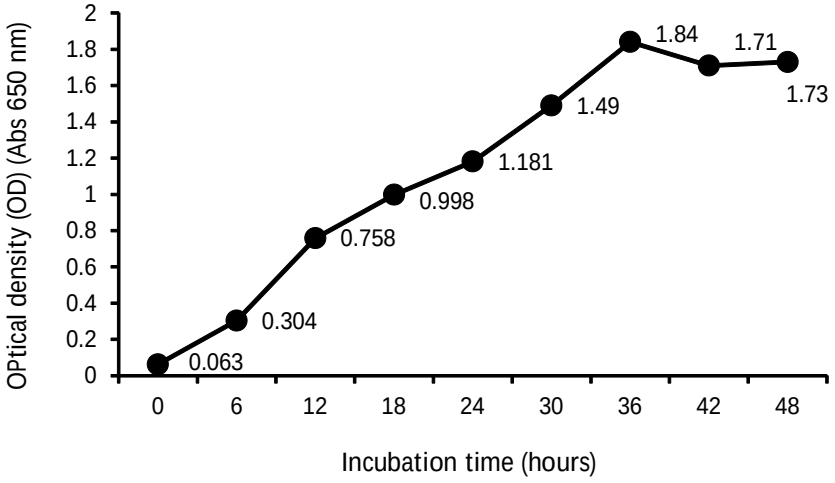

Figure 1. Growth curve of RGL1.1.

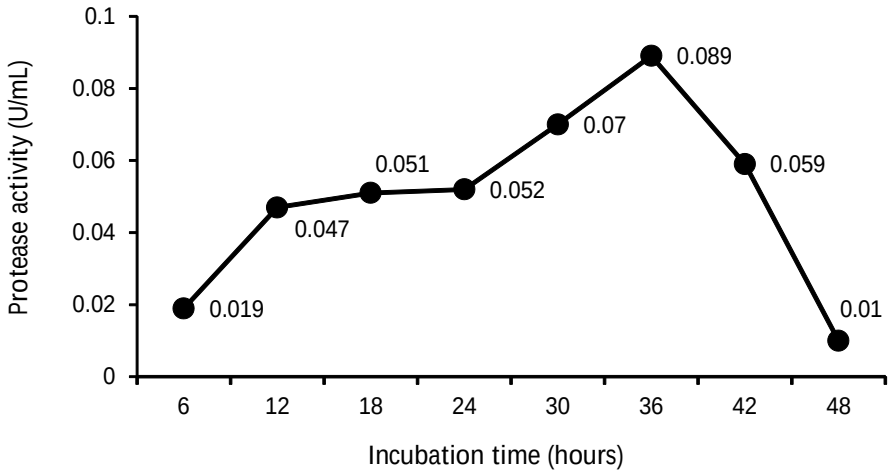

Figure 2. Proteolytic activity of RGL1.1.

isolate reached the optimum value at 50°C. At this temperature, the interaction between the enzyme and the substrate is very effective to allow the easier formation of enzyme-substrate, so it will increase the product (Nelson & Cox, 2005). The activity of protease enzyme of RGL1.1 isolate increased from 30°C to 50°C and decreased above 50°C. After passing the optimum temperature, enzyme activity generally decreases because the enzyme begins to damage the active group. According to Muchtadi *et al*. (1992) *in* Fathimah & Wardani (2014), enzymes have chemical bonds in the form of hydrogen, ionic/van der Waals bonds, and hydrophobic interactions that under normal circumstances maintain the structure of the enzyme. At high temperatures, this bond will break so that enzyme's proteins will denature causing the enzyme's active side to change conformation and reduce its catalytic activity. In addition, Montgomery (1993) *in* Fathimah & Wardani (2014) argues that at high temperatures, important bonding forces are damaged by the increasing thermal vibrations of atoms thus destroying the three-dimensional structure of the enzyme. Similarly, the substrate may undergo a conformational change where its reactive group will be inhibited when entering the active site of the enzyme.

Identification of Selected Proteolytic Bacteria

The biochemical and morphological characterization of RGL1.1 is presented in Table 6. Based on the microscopic observations, the RGL1.1 isolate had a rod-shaped form, lined up into long chains, and was a Gram-positive type of bacteria (Figure 5). The bacteria gave a positive result in the catalase test, which

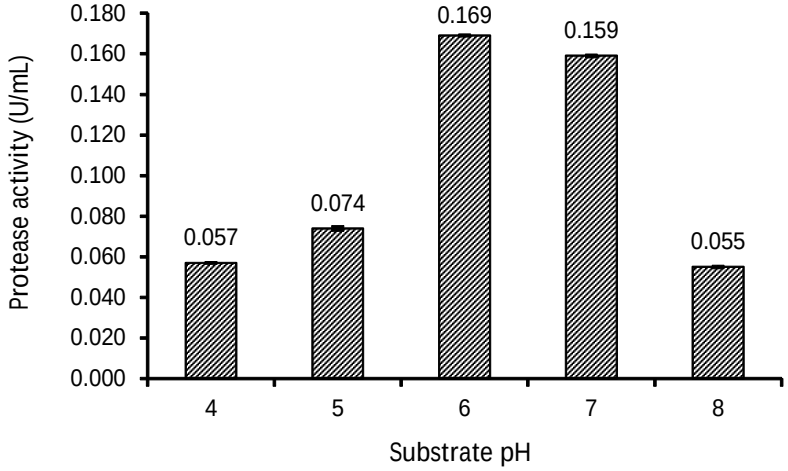

Figure 3. Protease activity (U/mL) of RGL1.1 isolate incubated at different pH levels.

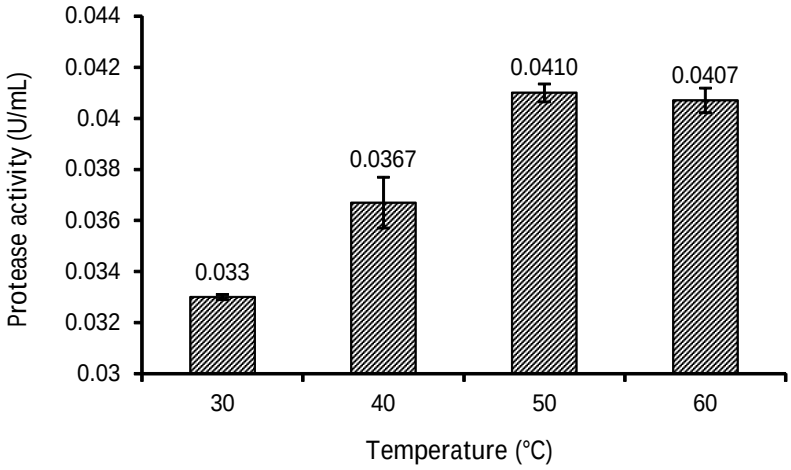

Figure 4. Protease activity (U/mL) of RGL1.1 isolate incubated at different temperatures.

showed that RGL1.1 isolate was able to live in aerobic conditions. Aerobic bacteria use catalase enzymes to detoxify hydrogen peroxide and superoxide radicals which are toxic by-products of aerobic metabolism (Martins *et al*., 2011). These bacteria also gave a positive result in oxidase enzymes test that indicates the bacterium has cytochrome oxidase which is a characteristic of aerobic organism (Trivedi *et al*., 2010).

In addition, RGL1.1 isolates were non-motile or immobilized characterized by the absence of flagella in the bacterial body. The bacteria also gave a positive result in gelatin test in which RGL1.1 isolate was able to hydrolyze gelatin and casein which are parts of protein.

The PCR results of RGL1.1 isolate were sequenced and analyzed using the EzTaxon server. The results showed that RGL-1.1 isolate had 99.93% similarities with *Bacillus cereus* (Table 7). *Bacillus cereus* is a rod-shaped bacterium, a Gram-positive, motile, and endospore bacteria (Hema & Shiny, 2012). This bacterium is potential to be used as a probiotic (de Souza *et al*., 2012) and can produce important enzymes such as proteases (Maal *et al*., 2009).

Protease is one type of hydrolase class enzyme that serves to break down proteins into simpler molecules, such as short oligo-peptides or amino acids by hydrolysis reactions in peptide bonds (Watanabe & Hayano, 1994). This enzyme is needed by all living things because it is essential in the process of protein metabolism and plays a role in helping protein digestion in food (Polaina & MacCabe, 2007). This study showed that RG1.1 isolates could potentially be used as protease-producing bacteria. This was indicated by its high protein activity and was not pathogenic to tilapia. The protease enzyme produced by RG1.1 bacteria can be used as a supplement in the feed (enzyme or probiotic) or to ferment certain fish feed raw materials. Several studies on the use of proteases have been done to improve

Table 6. Morphological and biochemical properties of RGL1.1 bacteria

Type of test	RGL1.1 isolate characteristics
Gram	Positive
Colony shape	Milky white, rounded jagged with core
Cell shape	Rod shape
Catalase	+
Oxidase	+
Motility	Motile
Gelatin	+
Peptonization	+

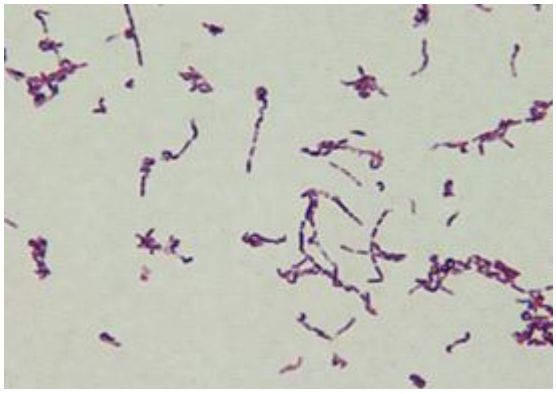

Figure 5. RGL1.1 isolate morphology.

the quality of feed and fish digestibility. For example, Drew *et al*. (2005) reported that proteases were able to improve nutrient and feed conversion of coextruded canola : peas feed than flax : peas feed. The same result was reported by Dalsgaard *et al*. (2012) where proteases were capable of improving nutrient digestibility from the soybean-based feed. According to Kemigabo *et al*. (2017), the addition of protease to catfish feed could increase the digestibility of the feed and an efficient protease utilization in feed could be reached if the feed contained 50%-55% of protein. Similarly, Irawati *et al*. (2015) and Ananda *et al*. (2015) reported that the addition of papain (protease) in artificial feeds had a significant effect on the relative growth, protein efficiency ratio, the feed efficiency of tilapia (*Orerochromis niloticus*) and catfish (*Pangasius hypopthalmus*). Besides as a supplement, protease can also be used to improve the quality of raw materials of fish feed. Mulia *et al*. (2014) reported that the fermentation of tofu waste with *Aspergillus niger* as proteolytic microbes could increase the levels of crude protein in the material. The use of an inoculum of 2.5 mL/50 g of tofu wastes was able to increase the crude protein content of the tofu from 14.93% to 27%. Samaddar & Kaviraj (2015) reported that the use of *Lactobacillus acidophillus* bacteria was an appropriate fermentation technique to improve the quality of animal blood for fish feed ingredient. The fermentation results showed an excellent protein quality containing several essential amino acids such as arginine, leucine, lysine, phenylalanine, threonin, and valine. The use of proteolytic bacteria (*Bacillus subtilis*) through fermentation can also improve the quality of poultry feather flour. Inoculum use of 10 mL/2 g of chicken feather flour may increase the crude protein content of this flour from 73.56% to 80.59% (Mulia *et al*., 2016). The utilization of protease-treated blood gave a positive effect on the digestibility and growth of the fish. For example, Palinggi *et al*. (2013) reported that tiger grouper fed with a diet containing blood meal after treated with protease were not significantly different in weight gain, specific growth rate, protein retention, and feed efficiency with fish fed with a non blood meal. This suggests that the use of blood meal with protease did not have a negative effect on the growth performance of tiger grouper. Based on these reasons, RG1.1 isolate has a great opportunity to be developed as a protease producer

Table 7. Closed bacteria identity based on EzTaxon server analysis

Sample code	Sequence code	Closed bacteria identityfrom homology result of EzTaxonserver [www.ezbiocloud.net/eztaxon/results]		
RG1.1	> contig_RG1.1	***Bacillus cereus* ATCC 14579(T)**		
		Accesion no.	:	AE016877
		Similarity	:	99.93%
		Length of alignment	:	1,512 bp
		Total nucleotide compared	:	1,343 bp
		Different nucleotide	:	1
		Completeness	:	100%

that can be utilized to improve the quality of fish feed and support better fish growth.

CONCLUSION

Based on the result of this research, it can be concluded that RGL1.1 isolate can potentially be utilized as protease producing bacteria. This isolate has been identified as a type of Gram-positive bacteria with rod shape that have 99.93% similarities with *Bacillus cereus*.

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