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Isolation and identification of rumen microbes and rumen fluid enzymes to use as the bio-degradator feed in aquaculture

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Abstract
The aim of this study was to isolate and identify a cellulolytic bacterium from the rumen cattle and proteolytic enzymes from the rumen sheep fluid. Biodegradation by cellulolytic rumen bacteria can be used as a source of cellulolytic bacteria and biodegradation by proteolytic enzymes that act to degrade feed fibrous material to improve the quality of nutrients and digestibility of feed ingredients at a cheaper price than the use of commercial cellulase enzymes. The cattle rumen fluid were used to identify the rumen microbes. Based on the cellulolytic analysis through Iodin test, two bacteria isolates were selected and showed the highest score of cellulolytic index scored of 3.0 and 3. Morphologically, the microbes identified as *Bacillus megaterium* and *Bacillus mycoides* and using KitStandard Analytical Pofile Index (API). In another side, the sheep rumen fluid were extracted to analyzed the enzymes activity and showed the rumen enzyme activity obtained in this study resulted much higher cellulase activity of 1.66 IU / ml / min than other enzymes such as amylase, phytase, and protease. The present study showed that rumen fluid has its possibility as bio-degradator of feed materials in aquaculture, which contain high level of cellulose.

Keywords: Cellulase, bacillus, rumen microbes, rumen enzyme, rumen fluid

Introduction
The limitation of fish in utilizing fiber is related to the limited availability of cellulotic enzymes in the digestive of fish. Excess fiber levels in fish feed at certain levels can inhibit the growth performance. Several studies report that in the digestive tract of fish found small amounts of cellulase activity [1-3].

Related to this, it is very important to find enzyme sources or microbes which produce the enzyme efficiently and effectively to improve the quality of aquaculture feed materials. One of the potential source is ruminal rumen fluid. Rumen fluid is a cheap source from the slaughterhouse waste and it obtained by squeezing the contents of the rumen. The availability is quite abundant and potentially as a source of microbes and enzymes. Ruminant stomach contains crude fiber such as cellulose, starch, and xylan as the basal feed for ruminants [4]. The rumen fluid contains the proteolytic microbes and enzymes [5] and can be use certainly as a source of the hydrolase enzyme [6].

Previous study [7] has reported that the feed crude fibers, which contain cellulose, were not completely converted to animal product in intensive animal farming. Microbial community including bacteria, fungi, and protozoa ferments these materials in the rumen. Rumen microbes secrete digestive enzymes into rumen fluid to help degrade food particles. Some microbes produce enzyme to degrade cellulose namely, cellulase. Rumen microbes have been identified in the production of cellulases include bacteria, some fungi, and actinomycetes (8). Cellulases bacteria such as Bacillus [9] has also been identified. Beside depends on the type of microbes, enzyme activity in rumen fluid also depends on composition and treatment of ruminant feeds [6]. Javiland, *et al.* [10] reported that the exogenous fibrilotic enzymes are very effective in reducing fiber content of raw materials feed such as rice straw and corn silage. Exogenous enzymes with multi enzyme contents are expected to be capable too.

Research on isolation and identification of ruminal microbes and fluid enzymes are still wide. In this study, the cellulolytic microbes were isolated from cattle rumen fluid and the potential the enzymes were hydrolyzed from sheep rumen fluid. Biodegradation product from ruminal rumen can be used to degrade fibrous feed materials for aquaculture production. So the quality of nutrients can be optimize.

Materials and Methods

Sample Collection

The experimental samples were Cattle rumen fluid and Sheep rumen fluid which obtained from slaughterhouse in Bandung and Bogor area. Pre-sterilized syringe and plastic bags were used for sample collection. The samples were taken in three replications from each rumen source in one take-up, covering the left, right, and middle rumen parts. This aims to make the sample homogeneous. The sample of rumen were stored at 4 °C for preservation reason. In addition, after being kept in cold condition, the sheep rumen fluid was centrifuged at a speed of 10,000 rpm for 20 minutes, then the supernatant formed was reacted using ammonium sulfate by a magnetic stirrer for approximately one hour and kept it for 24 hours at 4 °C. Then it was centrifuged with a speed of 10,000 rpm for 15 minutes at 4 °C, the deposit was taken as an enzyme source, dissolved in phosphate buffer and stored at 4 °C.

Isolation and Identification of Cellulolytic Microbes from Rumen Fluid of Cattle

The preparation consists of three stages; the isolation of aerobic microorganisms from cattle rumen fluid, which were found as cellulolytic and amylolytic, selection of microorganisms, determination and identification of selected microorganisms. The parameter observed from each isolated microorganism is the cellulolytic ability through the Iodine Test.

A sample of cattle rumen fluid (1 ml) was suspended with 9 mL sterile physiological NaCl(v/v) . Serial dilutions from $10^{-1}$to 10^{-5} were prepared. The first dilution started from 10^{-1} then 1 mL taken and put into the second tube. After the dilution were completed, an aliquot of 1 mL of each dilution from 10^{-3}, 10^{-4}, and 10^{-5} was inoculated into a sterile petri dish with Sabouraud dextrose agar (SDA) and Medium Cellulose (CMC), in duplicate. Plates were incubated at 37 °C for 48 h.

Screening of Cellulolytic Microbes from Rumen Fluid of Cattle

Two colonies with maximum clearing zone were isolated for further screening. The isolate selected as candidates were used at a later stage of the study. The isolate were morphologically identified and determinated using the 50 CHL KitStandard Analytical Pofile Index (API) 50 CHL Kit (Bio Meriueux, Marcy l'Etoile, France, 2006).

The test of cellulose enzyme activity from sheep rumen fluid

The extract sheep rumen was dissolved phosphate buffer liquid in a ratio of 1: 1, then being tested for the activity of cellulase, amylase, protease and lipase enzymes. The test of cellulase / FP-ase enzyme activity were carried out according to the Ghose method [11].

Results

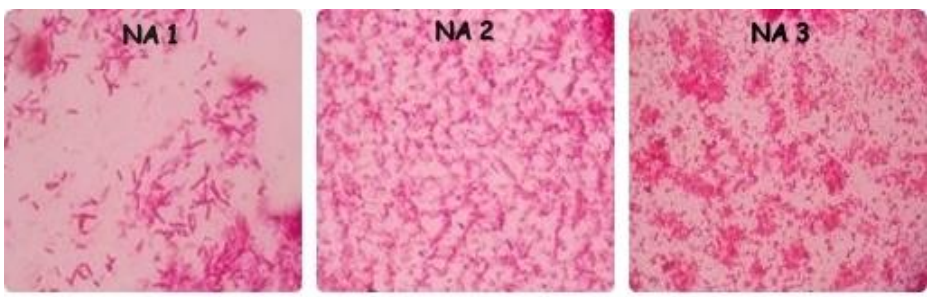
Isolation and screening of cellulolytic bacterium

The microbes observed in this study were found as a bacterium. Thirteen colonies of cellulolytic bacteria were isolated from the rumen fluid of cattle and cellulolytic activity quantitatively screens using Congo red staining 0.1%, namely, NA 1, NA 2, NA 3, NA 4, NA 5, MRS 1, MRS 2, MRS 3, CM 1, CM 2, CM 3, CM 3, and CM 5. The bacterium was isolated on pH 6.0-6.5 and temperature 37 °C for 24 h.

Table 1: Cellulolytic index of microbes from cattle rumen fluid

Medium	*Code*	Clear zone diameter (cm)	Colony diameter (cm)	Cellulolytic index
Cellulose Medium (CM)	CM1	0.80	0.40	2.00
	CM2	1.20	0.40	3.00
	CM3	1.60	0.60	2.67
	CM4	0.90	0.60	1.50
	CM5	1.40	0.40	3.50
Natrium Agar (NA)	NA1	1.20	0.70	1.72
	NA2	1.70	1.30	1.31
	NA3	2.40	2.20	1.09
	NA4	1.20	0.80	1.50
	NA5	1.30	1.10	1.18
Man, Rogosa and Sharpe (MRS)	MRS1	0.40	0.40	1.00
	MRS2	0.60	0.60	1.00
	MRS3	0.80	0.30	2.67

Based on the cellulolytic analysis through Iodin test, two bacteria isolates were selected and showed the highest score of cellulolytic index, code CM2 and CM5 scored of 3.0 and 3.5 with the clear zone diameter 1.20 cm and 1.40 cm (Table. 1). The isolate has greater cellulolytic activity than >1.0 as an indication of high cellulolytic activity (12). The Morphology of cellulolytic bacteria isolates showed in Fig. 1.



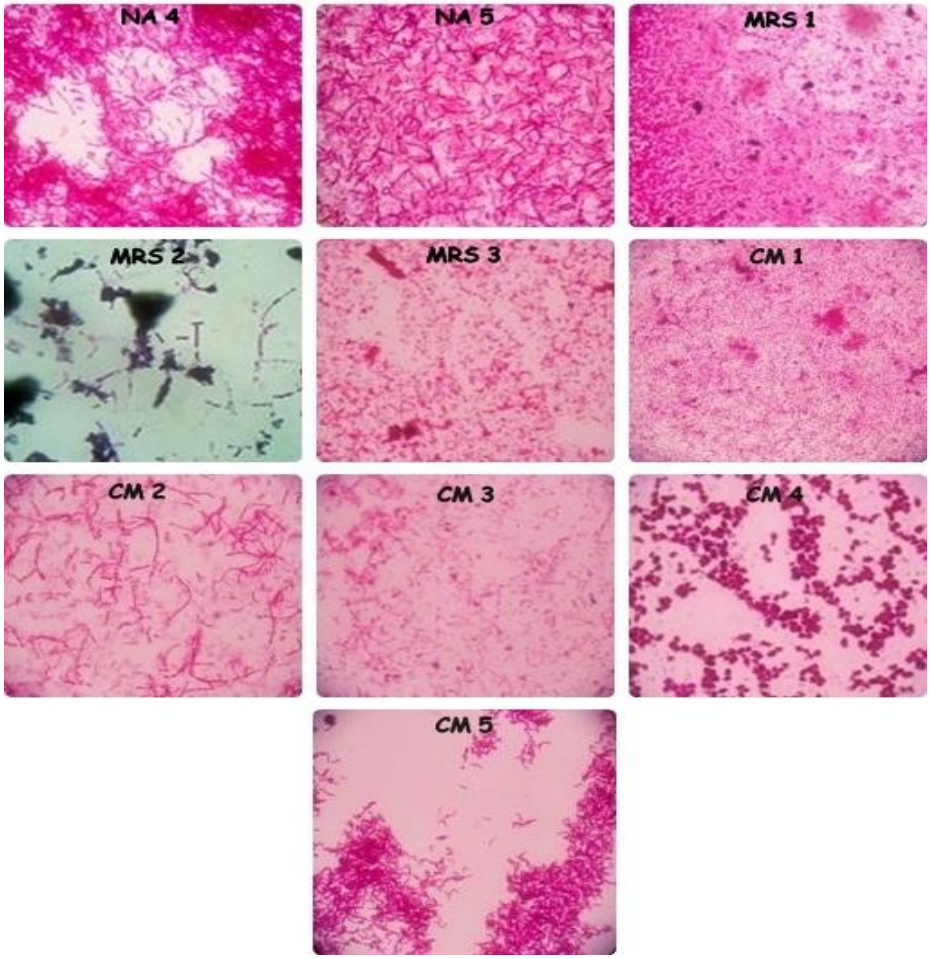


Fig 1: Gram staining of the cellulolytic bacterium

Macroscopically, the CM2 was found rounded, smooth, and milky white color, while the CM5 was found flat shaped, somewhat rough, and milky white. Specifically, these two bacteria were bacilli, Gram positive, and spores. Meanwhile, microscopic identification results indicated that the bacteria were thought from the genus *Bacillus*. The screening of the next identification was carried out using API Test CHB 50. The results obtained after 48 hours of API Test were identified that CM2 was *Bacillus megaterium* and CM5 was *Bacillus mycoides* (Figure 2).

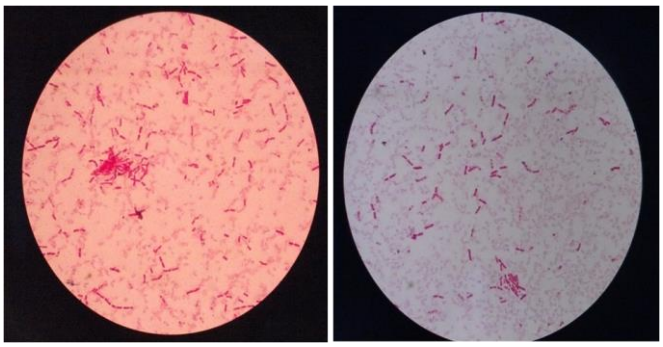


Fig 2: *Bacillus megaterium* (left) and *Bacillus mycoides* (right). Magnification 1000 times, screening of the Iodine test to the species level identification was carried out macroscopically and microscopically using a moist chamber.

Hydrolysis Enzyme activity test of extract sheep rumen fluid

The results of enzyme activity test of amylase, protease, lipase, cellulase and phytase in sheep rumen fluid are presented in Figure 3. It showed that Cellulase activity was greater than others with the values of 1.66 ± 0.19 IU / ml / min; amylase 1.32 ± 0.02 IU / ml / min; phytase 0.27 ± 0.13 IU / ml / min; protease 0.26 ± 0.07 IU / ml / min; lipase 0.01 ± 0.00 IU / ml / min.

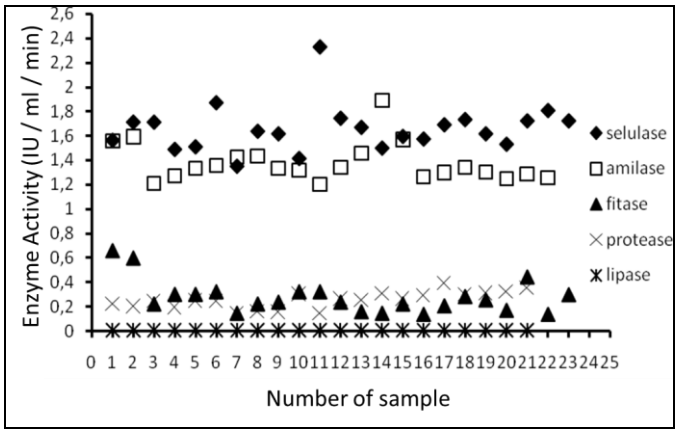


Fig 3: The enzyme activity test of amylase, protease, lipase, cellulase and phytase in sheep rumen fluidextract.

*The substrate used: cellulose test (filter paper); amylase test (starch); protease test (casein); lipase test (olive oil); phytase test (phytic acid)

Discussion

Rumen microbes secrete digestive enzymes into rumen fluid to help degrade food particles. Microorganism and Enzyme activity in rumen fluid also depends on food composition or treatment [6]. Accordance with some of the previous study

which found isolates from the genus *Acidothermus, Bacillus, Cellulomonas, Cellvibrio, Cytophaga*; while Chusniati [13] isolated the genus *Nitrosomonas, Acidothetmus, Bacillus, Cellulomonas, Cellviomonas, Cellvibrio, Cytophaga*. In this study, the selected bacteria from cattle fluid was from genus *Bacillus*.

One of the requirments of microbes as feed biodegradators is to be classified as aerobic facultative microorganisms, because the process of biodegradation of feed ingredients will run aerobically. Selected microbes *Bacillus megaterium* and *Bacillus mycoides* from cattle rumen fluid could survive in aerobic condition. Beside microbes, this study also evaluated the hydrolytic enzyme activity from rumen fluid. The selected rumen fluid came from sheep rumen because its potentiality as a source of hydrolysis enzymes such as amylase, protease, lipase and cellulase and make it one of the sources of alternative supplements and can be utilized certainly to improve the quality of feed material in aquaculture. Previous study [6] reported that sheep rumen fluid containing microbes which produce higher cellulase, amylase, protease and lipase enzyme activities than rumen fluid without microbes with enzyme activity was0,03 (IU/ml/min). Comparing to the enzyme activity reported by previous study [6], the rumen enzyme activity obtained in this study resulted much higher cellulase activity of 1.66 IU / ml / min. The difference in value of this activity is thought to be due to the type of food consumed by ruminant during the maintenance period. The sheep in this study were conditioned only by eating leguminosae mixed with other types of green. Song *et al*. [14] reported that the cellulases from rumen bacteria retain 70% of its activity in the pH range from 5 to 7 and in a temperature range from 30 °C to 50 °C

It was reported that enzyme activity in the rumen is very closely related to the diversity of microorganisms in the rumen. Where cellulase enzyme activity is obtained from the rumen microbes that secrete cellulase enzyme into rumen fluid to help degrade feed particles that contain cellulose. The microbes in the rumen can hydrolyze cellulosic polymers, so the energy from the complex polymers would be delivered to the host [4]. This potentiality could help the problems in aquaculture as bio-degradators of plant-based feed materials, which contain high level of cellulose. As it known that both cattle and sheep rumen can easily obtained from animal slaughtered house

Conclusion

In conclusion, the cellulolytic bacteria have been successfully isolated from cattle rumen fluid with cellulolytic activity scored 3.0 and 3.5, it was identified came from genus *Bacillus*. Besides that, this study also investigated the potentiality of extract enzyme from sheep rumen fluid and showed that the cellulase activity (1.66 ± 0.19 IU / ml / min) was higher than other enzymes in sheep rumen.

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