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Screening, isolation and selection of cellulolytic fungi from cattle rumen fluid for bio-degradator in aquaculture

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Abstract
Screening and isolation of cellulolytic fungi were done using cattle rumen fluid conducted at a local slaughtered house in Bandung, West Java, Indonesia. This research was aimed to isolate cellulolytic fungi with over production of cellulase components. Isolated fungi had shown the ability to degrade cellulose base on decolorization of CMC selective agar using Grams iodine as color indicator. Two isolates with cellulolytic activity with the result of CM Case 2.33 and 1.24, and FP-ase 0.30 and 0.11 were identified as *P. nalgiovense* (SpC.PD) and *A. tamarii* (SpF.PD). This study identified those species as highly cellulolytic in producing cellulase activity. Identification of the cellulase enzymes can help industries to exploit this enzyme for feed bio-degradator such as in aquaculture production.

Keywords: Cellulase, cellulase fungi, cellulolytic, *P. nalgiovense*, *A. tamarii*

1. Introduction
The cellulose is a linear polymer of glucose form the basic structure of the stem cell of the plants. The plants contain of cellulosic polysaccharides and hemicellulose up to 50% of the biomass, which can produce cellulases enzymes to degrade the linear polymer glucose of cellulose. The high content of cellulases enzymes is very important in the process of bioconversion of cellulose into beneficially products [1].

Fungi, bacteria and actinomycetes can produce cellulase. Fungi are considered as a more active cellulose decomposer and is the main cellulase in producing microbe [2]. *Trichoderma viride* for example, it can convert natural cellulose into glucose [3] because having a complete cellulase complex, while *Bacillus* was unable to produce the complete cellulose complex [4]. Additionally, despite cellulases production by bacteria takes shorter than *Trichoderma*, but enzyme activity is slower [5].

Related to this, aquaculture sector needs to improve the quality of feed materials and the enzyme from the microbes are quietly efficient and effective. Nevertheless, the source of cellulolytic fungi is limited due to the availability of plants fungi with high content of cellulolytic activities. Therefore, to overcome this problem, some source of cellulolytic fungi such as rumen fluid might be use.

A complex mixture of food fragments, water and microorganisms characterizes are suitable in the rumen environment. At this site, there are populations of bacteria, ciliated protozoa, aerobic fungi, mycoplasma and bacteriophage, which establish various positive or negative interactions between themselves [6].

Furthermore, that the microorganisms carried from the feed, especially the types of fungi, were able to survive in the rumen conditions, and some types were still work functionally in the digestion of ruminant [7].

2. Material and Methods
2.1 Sample collection
Cattle rumen fluid samples were collected from slaughterhouse in Ciwastra area, Bandung, West Java, Indonesia. Pre-sterilized syringe and plastic bags were used for sample collection. The samples were taken three times from each rumen source, the part of left, right, and middle side of cattle.

The sample of rumen fluid brought into the laboratory for analysis purpose. All laboratory handling of rumen fluid was carried out under continuous flushing with CO_2.

2.2 Fungi Isolation
The sample was put in a sterile petri dish, then the Agar medium was added into the cellulose medium, namely, Potato Dextrose Agar and Sabouraud Dextrose Agar with 1% CMC, as much as 15-20 ml per petri dish. Subsequently, amoxicillin was assorted into each petri dish aseptically which already contained the diluted sample solution, then homogenized with a circular motion and being kept under frozen condition. After the medium had frozen, the petri dishes wrapped using the paper and incubated at room temperature for three days or until fungi colony was being visible.

2.3 Microbes Selection
Potato Dextrose Agar (PDA) and Sabouroud Dextrose Agar (SDA) were mixed with 1% CMC and 20 ml cellulose medium. At that time, it added aseptically into a petri dish and stored at low temperature until frozen. Then a loop of fungi was put in the middle of the medium. Subsequently incubated for 72-96 hours at 28ºC. After the incubation was being completed, the iodine solution was dripped and homogenized on the agar surface. Iodine will react with cellulose in the medium to cause a blue color. The diameter of the fungi and the diameter of the clear zone formed around the tested mold colonies were measured. The fungi with the highest clear zone diameter value is the best mold in producing cellulase enzymes, so it was being used as an inoculum in fermentation. The identification of the two types of fungi which has the largest clear zone was done using a Moist Chamber.

2.4 Data Analysis
The fungi isolate from cattle rumen fluid were previously stained with the Gram technique and grouped according to the physiological and morphological profiles [8]. The morphological features such as thallus morphology, growth patterns and position of sporangia, were examined macroscopically and microscopically [9].

Screening of Cellulolytic Fungi from Rumen Fluid of Cattle
The parameters observed in this study were the identification of fungi which has the greatest cellulolytic activities obtained from cattle rumen fluid. The isolate selected as candidates were used in the next step of the study. The isolate were morphologically identified and determinated using the 50 CHL KitStandard Analytical Profile Index (API).

2.5 Cellulolytic Activity Enzyme Test
CMC-ase activity is determined according to the method of HAGGETT *et al*. [10] with modification. One unit of enzyme activity is defined as the number of enzymes that yield 1 µmol glucose per minute under essay conditions, CMCase against CMC substrate.

The filter paper activity (FP-ase) is determined according to the method of Mandels *et al*. (1976) uses Whatman No.1 filter paper measuring 1 cm x 6 cm. The reaction incubation time is 60 minutes. Determination of reducing sugars using DNS. One unit of enzyme activity according to references in literature [11].

3. Results
3.1 Isolation and Screening Cellulolytic Fungi
Seventeen fungi colonies were isolated from cattle rumen fluid to observe cellulolytic activity, Namely, Sp1_SD, Sp2_SD, Sp3_SD, Sp4_SD, Sp5_SD, Sp6_SD, Sp7_SD, Sp1_PD, Sp2_PD, Sp3_PD, Sp4_PD, Sp5_PD, Sp6_PD, Sp7_PD, Sp1_CM, Sp2_CM, and Sp3_CM. The isolates were identified on Sabouraud Agar (SDA), Potato Dextrose Agar (PDA), and Cellulose Medium (CM). The results showed that all isolates from the cattle rumen fluid gave positive results (Table 1).

Table 1: The clearing zone and colony diameter of fungi isolated from cattle rumen fluid

Medium	Code	Clear zone diameter (cm)	Colony diameter (cm)
Sabouraud Agar (SDA)	SpA_SD	4.90	4.70
	SpB_SD	4.90	4.70
	SpC_SD	1.00	1.00
	SpD_SD	1.00	0.70
	SpE_SD	1.10	0.90
	SpF_SD	0.80	0.60
	SpG_SD	1.10	0.90
Potato Dextrose Agar (PDA)	SpA_PD	2.30	1.20
	SpB_PD	1.30	1.20
	SpC_PD	2.10	0.90
	SpD_PD	1.30	1.30
	SpE_PD	1.00	1.00
	SpF_PD	1.10	0.90
	SpG_PD	1.00	0.90
Cellulose Medium (CM)	SpA_CM	1.60	1.50
	SpB_CM	1.20	1.10
	SpC_CM	3.00	3.00

Each isolate of microorganisms that was isolated had a different average clear zone diameter and colony diameter (cm), as shown in Table 1. The highest clear zone was SpA_SD and SpB_SD with clear zone diameter 4.90 cm and colony diameter 4.70 cm. The isolate has greater cellulolytic activity than >1.0 as an indication of high cellulolytic activity [12]. Two fungi isolate were selected based on higher values, namely, SpC. PD and SpF. PD with the cellulolytic index values of 2.33 and 1.24, respectively (table 2).

Table 2: The cellulase activities of fungi isolated from cattle rumen fluid used in cellulolytic screening study (CMCase and FPase)

Medium	Code	CMCase (U/ml)	FPase (U/ml)
Sabouraud Agar (SDA)	SpA_SD	1.04	0.03
	SpB_SD	1.04	0.03
	SpC_SD	1.00	0.04
	SpD_SD	1.22	0.07
	SpE_SD	1.22	0.09
	SpF_SD	1.20	0.09
	SpG_SD	1.22	0.10
Potato Dextrose Agar (PDA)	SpA_PD	1.92	0.23
	SpB_PD	1.08	0.10
	SpC_PD	2.33	0.30
	SpD_PD	1.00	0.04
	SpE_PD	1.00	0.13
	SpF_PD	1.24	0.11
	SpG_PD	1.11	0.04
Cellulose Medium (CM)	SpA_CM	1.07	0.06
	SpB_CM	1.09	0.09
	SpC_CM	1.00	0.10

In this study, after fungi isolates selected, the identification into species level was carried out macroscopically and microscopically using a moist chamber. The key book for

fungi identification, "*Introduction of Food Borne Fungi*", showed that the SpC_PD species is *Penicillium nalgiovense* and SpF_PD is identified as *Aspergillus tamari* (Figure 1).

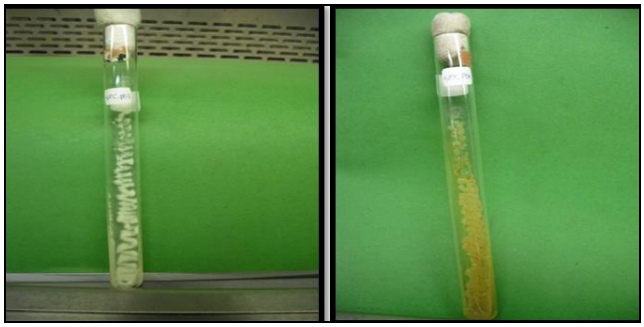

Fig 1: Microscopic Identification of *P. nalgiovense* (left) and Bacillus *A. tamarii* (right). Magnification 400 times, screening of the Iodine test to the species level identification.

The results of macroscopic identification can be described as follow *P. nalgiovense* has a milky white, its spores resemble powdered milk, initially white after aging is getting less bright. *A. tamarii* is macroscopically yellow-brown in color, at the beginning of rejuvenation has a bright yellow and after a few days the color changes into sharp contrast to dark brownish yellow.

Based on the identification key, *A. tamarii* forms yellow colonies, yellow-brown conidial heads, yellow or brown non-columnar conidial heads, brown conidial heads significantly ornamental, whereas *P. nalgiovense* the colony was white and the conidiophores were smooth (Figure 2).

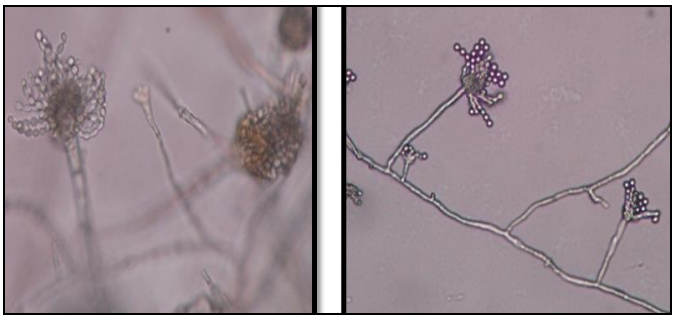

Fig 2: Macroscopic Identification of *P. nalgiovense* (left) and Bacillus *A. tamarii* (right). Magnification 400 times, screening of the Iodine test to the species level identification

4. Discussion

In the intestinal tract, the fungi contribute in association with bacteria and protozoa, to hydrolyze the fiber in diets, resulting of fermentation product that can be used by the host as sources of nutrition [13]. Some of gut fungi could survive in an aerobic condition and may be having a potentiality in aquaculture feed biodegradation. The environmental condition for microorganisms is very important to produce energy metabolism, namely oxygen. The previous study [14] were found that *Bacillus megaterium* and *Bacillus mycoides* could be the potential microbes biodegradator from rumen fluid. Beside bacteria, the potentiality of fungi as a cellulolytic are still invenstigated.

The research about cellulolytic fungi were concentrated mainly on soil fungi [15] for the cellulolytic activity. The source might be contain the microorganisms (bacteria, fungi and actinomycetes) whose capability and activity depend on the conditions to which substrates are exposed [16]. In this study, fungus isolation was done from different sources, such as cattle rumen fluid. The selected microorganisms, *P. nalgiovense* and *A. tamarii* are gut fungi that can survive in aerobic culture conditions on the medium of PDA. Moreover, the most common and most effective cellulase producers are *Trichoderma reesei, T. koningii, Fusarium* sp., *Aspergillus*, sp. and *Penicillium* sp. [17]. During the isolation process, these fungi were survived and had the capability to reproduce in selective medium. The aim of selected aerobic fungi is to make its capable as a feed bio-degradator in aquaculture.

Furthermore, it is known that the selected microorganisms have cellulolytic ability, which can be measured through the activity of the cellulase enzyme. According to previous studies, *A. tamarii* was reported contains amylase and glucoamylase enzyme activity [18], while *P. nalgiovense* has shown a high cellulase activity, 0.027U/ml [19]. Nevertheless, *A. tamarii* revealed the lowest hydrolytic activity on pectin [16]. Regarding this, the usage of cellulase enzyme is recently used as bio-degradator in aquaculture to support the feed digestion in aquatic animals and increase the growth performance.

5. Conclusion

The results obtained during this study indicated that cellulase activity of testing fungi isolated from cattle rumen fluid, *P. nalgiovense* and *A. tamarii* and were found relatively higher than other isolates from cattle rumen fluid with the cellulolytic index values of 2.33 and 1.24, respectively. This research revealed that the isolated fungi from cattle rumen fluid could survive in aerobic condition on the PDA medium and has a possibility as a biodegradation in aquaculture such as in fermentation processes.

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