

## Testing of Bacterial and Fungal Isolates from Rumen Fluid Used in Inoculants in the Fermentation of Feed from Agro-Industrial Waste

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### ABSTRACT

The addition of the inoculant can accelerate the fermentation process and improve the quality of the feed material in fermentation. This study aimed to isolate, test and select the rumen microorganisms that have high ability to degrade the fiber and improve the quality of materials used in inoculants to ferment agricultural wastes as alternative feed. Microbial isolate was extracted from cattle rumen fluid. The isolates type of fungus were grown on Potato Dextrose Agar (PDA) while the bacteria was grown on Nutrient Agar (NA) medium. Microbes of fungi were tested and selected based on their ability to produce ligninase, cellulase and hemicellulase enzymes. Bacteria were grown on MRS broth media as a confirmation for LAB colonies, then tested for gram staining, catalase test, growth on different NaCl levels (4 and 6.5%) and growth at various pH (3, 4, and 5). The results showed that five fungal isolates had a growth diameter of 0.1–0.37 mm on lignin, cellulose and hemicellulose media after seven days. Eleven bacterial isolates were indicated as lactic acid bacteria; almost all isolates could grow on NaCl 6.5%, but only four bacterial isolates could grow on NaCl 4% and five bacterial isolates could grow at pH 3. In conclusion, three isolates of fungi have a remarkable ability to degrade lignin. Five isolates of lactic acid bacteria have a comprehensive ability as a preservative in fermentation, so eight microbial isolates from rumen fluid can be used as inoculants in fermenting the feed material from agro-industrial waste.

**Keywords:** rumen fluid microorganism, fiber decomposing fungi, lactic acid bacteria.

### INTRODUCTION

Livestock productivity is determined by the quality and quantity of available feed, especially during the dry season. Low crude protein and high crude fiber in feed causes that the basic living needs of livestock are not fulfilled, thus reducing the efficiency of feed and animal health. Meanwhile, the remaining agricultural, plantation and food processing industries are abundant. The abundant by-product of the agricultural industry is the advantage of feed in Indonesia, because the price is relatively cheap, it is wide-spread and not

used by humans as food, but contains high lignocellulose and low crude protein. Dashtban et al., (2009) stated that lignocellulose is an organic component, which consists of three polymers, namely cellulose, hemicellulose and lignin. The low nutrient content of feed ingredients from agricultural waste can be increased by processing it into quality feed that increases livestock productivity.

The basic principle of increasing lignocellulosic digestibility is the release of lignin bonds with cellulose, hemicellulose, protein and other compounds, to increase the digestibility value of waste using appropriate microbes through a process of

enrichment, isolation, selection and identification (Bachruddin, 2014). On the basis of the consideration that ruminants are able to convert low-quality to high-quality feed due to the role of lignocellulotic microbes, it is important to isolate it to obtain rumen microbial isolates (bacteria and fungi) that have high ability to digest crude fiber in feed from agricultural, industrial and plantation waste. Before using agro-industrial waste as feed, it is necessary to enrich its components and increase the quality through biological treatment, with a fermentation process using rumen microbes as inoculants.

Cows rumen fluid has the potential as a feed additive because it contains a large microbial population. Of all rumen microbes, fungi are microbes that have the ability to degrade lignified cell walls (Abdullah et al. 1990), which is about 8% of fungi from the total microbial mass (Orskov and Ryle, 1990). Ruminants consuming forage with a greater proportion have a population of fungi in the rumen from  $10^4$  to  $10^5$  per ml of rumen fluid. Cattle have high digestibility of fiber, so it is possible that the rumen fluid contains lactic acid bacteria which have the potential to be biopreservative and inoculant in fermentation of fibrous feed ingredients (Nurhalimah et al, 2015; Jasin and Bachrudin, 2013). The digestive tract of ruminants (cows) is known to have microorganisms; the colon is a place of abundance of LAB, especially *Bifidobacterium* spp, estimated at  $10^8$ – $10^{11}$  per gram (Salminen and Wright, 1998).

Rumen microbes work in a mixed system, unable to work in a pure system alone (Van Soest, 1994). Yokohama and Johnson (1988) added that the interactions between microbes affect the presence of these microbes in the rumen. The fermentation of complex carbohydrates, such as cellulose, can be increased by the interaction between microbes (Baldwin and Allison, 1983). Fonty (1991) states that mixed fungal populations can digest up to 60% of plant tissue. Mixed protozoan populations without bacteria can digest 7% cellulose. Mixed bacterial populations can digest 40% cellulose during the same time, if both are present in optimal proportions will increase the ability to digest cellulose by up to 60% (Orskov and Ryle, 1990).

Processing of feed by fermentation or silage can use a fermenter inoculum derived from only one type of strain (single) or consortium (mixed inoculum) aims to accelerate the fermentation process and yield the best results. Inoculum (starter culture) can be obtained from commercial products

or from the results of self-isolation. The use of additives in feed can improve the silage quality (Parakkasi, 1999). The purpose of additives in silage making is to accelerate the formation of lactic and acetic acids to prevent the formation of undesirable fermentation, as well as a nutritional supplement in fermented feed. In order to avoid unsuccessful fermentation, it is recommended to add a homofermentative lactic acid bacteria inoculum (LAB), to ensure the occurrence of lactic acid fermentation. LAB is the most popular additive compared to acids, enzymes or others (Bolsen et al., 1995). Because of the importance of using inoculants in accelerating the fermentation process in improving the quality of feed from agro-industrial waste, it is necessary to isolate microbes from decomposing fungi and bacteria that produce lactic acid from the rumen fluid. Isolation and testing were conducted to obtain the fungal isolates that have a high ability to degrade fiber and lactic acid-producing bacteria which can be used as inoculants in fermenting agricultural waste as alternative feed.

## MATERIALS AND METHODS

### Source of microbes

The rumen fluid used as a source of microbes was taken from the Slaughterhouse in Makassar City. The dilution process was carried out by taking 1 ml of rumen fluid and suspending it into 10 ml of sterile distilled water, shaking it and making a  $10^{-1}$  to  $10^{-9}$  dilution series into a separate test tube. The concentration of the suspense sample was taken in one ml each and then poured into a petri dish containing PDA (Potato Dextro Agar). Chloramphenicol medium was used to grow fungi, while NA (Nutrient Agar) medium and cyclohexamide were employed to grow bacteria in a petri dish. The samples grown in PDA media were incubated for 3–7 days at room temperature, while the samples grown in NA were incubated for 24–48 hours (Agustini 2011). Every single colony that grew on the isolation plate, was immediately subcultured to new media to obtain pure culture. A single dish contained only one microbial colony.

### Isolation and testing of fiber-decomposing fungi

Screening of ligninase-producing isolates. Testing of ligninase-producing isolates aimed to

test the presence of lignin (ligninase) degrading enzymes, namely lignin peroxidase and laccase, a spot test was carried out by dropping a 1.0% pyrogallol solution mixed with 0.4% H<sub>2</sub>O<sub>2</sub> (1: 1). The brownish yellow color of the pyrogallol reagent drops indicates the activity of the enzyme lignin peroxidase. The laccase enzyme was detected using 0.1 M 1-naphthol reagent diluted with 96% ethanol. Purplish red color is a positive indicator of the laccase enzyme (Stalpers, 1978).

Screening of cellulase-producing isolates. Testing of cellulase-producing isolates using selective carboxymethylcellulose (CMC) agar, containing 0.2% NaNO<sub>3</sub>, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.05% MgSO<sub>4</sub>, 0.05% KCL, 0.2% sodium salt CMC, 0.02% peptone and 1.7% Difco agar. The formation of a transparent zone around the fungus colony identifies the activity of the cellulase enzyme produced by the fungus (Kasana et al., 2008).

Screening of hemicellulase-producing isolate. Testing of hemicellulase-producing isolates was carried out by culturing pure isolates aged 7 days into Czapek medium (Difco™) enriched by providing 0.2% xylan as a single carbon source (Zhang et al., 2005) and incubated at 30°C for one week. After 7 days of incubation, the clear zone area around the fungal colony was observed and measured. The formation of a transparent zone around the fungal colony identifies the activity of the hemicellulase enzyme produced by the fungus.

The enzyme activity was tested to determine its ability to degrade fiber (lignocellulose) qualitatively (Coughlan, 1988). Selection was based on the ratio of the highest clear zone to the diameter of the colonies planted (Kluepfel, 1988).

### Isolation and testing of lactic acid bacteria

Bacterial growth on selective media de Mann Rogoss Sharpe (MRS) Agar refers to Suardana et al. (2009). Testing and identification were carried out as a confirmation stage for LAB colonies growing on MRS broth media. Morphological identification was performed by observing isolates growing on MRS media taking into account the colony shape, elevation, edges and color of the bacterial colony.

### Gram test for bacteria

Mixing one loop of bacterial isolate was performed on a slide that were dropped 10 µL of 3%

KOH;. If mucus was formed, the bacteria were grouped into Gram negative, but if no mucus was formed the bacteria were grouped into Gram positive (Kurnia et al. 2016).

### Test for bacterial growth at different NaCl levels (4% and 6.5%)

Bacterial isolates were inoculated on MRS broth media that had been added with NaCl with a concentration of 4% and 6.5% and then incubated at 35 °C for 24 hours, the growing bacteria were marked by the turbidity of MRS broth media (Susilawati, 2016).

### Test for bacterial growth at various pH values (pH 3, 4 and 5)

The bacteria grown in MRS broth that has been set pH using concentrated HCl (acid) and NaOH (wet) were homogenized using a vortex and incubated at a temperature of 39 °C for 48 hours (Hadioetomo, 1985), the bacteria that grew were marked by media turbidity, and the level of turbidity was measured using a spectrometer.

### Enzyme test (cellulase, chitinase and pectinase)

Enzyme tests using CDA media (Czapex dox broth) added with cellulase, chitinase and pectinase enzymes were adjusted to the test; one loop of bacterial isolate was inserted into the media and incubated at room temperature for 48 hours. The enzyme-producing bacteria were marked by a clear zone on the edge of the bacterial colony.

## RESULTS AND DISCUSSION

### Testing of fungi isolated from rumen fluid

The results of the observation were 16 isolates of fungi from rumen fluid that could grow on PDA media. The isolates were purified and tested for growth on lignin media as the initial stage of selection, then their growth diameter on a petri dish was observed, and 5 best isolates were obtained. Furthermore, the 5 selected isolates were tested for their ability to produce ligninase, cellulase and hemicellulase enzymes. The growth diameter of 5 fungal isolates in producing enzymes can be seen in Table 1.

**Table 1.** Average growth diameter of fungal isolates on lignin, cellulose and hemicellulose media

No	Sample Code	Growth diameter (mm)		
		Lignin	Selulosa	Hemiselulosa
1	Cr 10 <sup>1</sup> 1	0.20	0.20	0.30
2	Cr 10 <sup>4</sup> 3	0.23	0.13	0.20
3	Cr 10 <sup>3</sup> 2	0.35	0.20	0.20
4	Cr 10 <sup>1</sup> 3	0.30	0.37	0.20
5	Cr 10 <sup>2</sup> 3	0.33	0.15	0.30

In the lignin test, the largest clear zone of the Cr 1032 isolate, indicated that it had a higher potential to produce ligninase enzymes. The higher the resulting lignocellulolytic index, the greater the enzyme produced by the fungal colony. The size of the lignocellulolytic index shows the size of the isolate's ability to degrade lignocellulose (polysaccharide compounds). The resulting lignocellulolytic index reflects the activity of enzymes in degrading lignocellulose.

Selection of fungal isolates was performed through testing the ability of the fungus to degrade fibers in a medium containing CMC, xylan, lignin and mannan. They were selected to produce the fungal isolates able to utilize the existing carbon source (CMC, xylan, lignin and mannan). It can be seen from the clear zone formed around colonies of fungi area. The clear zone formed indicates that the fungal isolate produces cellulase, xylanase, manannase and ligninase enzymes to degrade lignocellulose. The potential isolates of lignocellulolytic fungi were shown by the wide clear zone formed and the high hydrolysis index in the broth medium containing lignocellulose (CMC, xylan, mannan and lignin).

The test results showed that the differences in species of fungi affect the size of the enzyme activity which is derived from the ratio of the clear zone (lignocellulolytic index). In accordance with the statement of Kusnadi (2010), the ability to produce enzymes in each fungus is specific to species and even strains.

In Table 1 it can be seen that 5 fungal isolates produce clear zones in cellulose testing, indicating that these isolates have the potential to be cellulase-producing fungi. On the basis of the qualitative test results, it was found that the average diameter size of the clear zone in isolates was from the smallest 0.1 mm to the largest 0.37 mm size. The size of the clear zone is an early indication of cellulase produced. The larger the clear zone produced, the greater the possibility

of cellulase produced or higher enzyme activity. Observations of white fringe fungi isolates were conducted by Hartana (2014), who reported that the colony was light colored with mycelium like cotton. According to Adlini (2014), *Aspergillus* is a type of fungus that can grow on media that contains cellulose. According to Jalil (2004), *Aspergillus* can degrade cellulose and produce cellulase excreted at the tips of the hyphae to degrade the polymer nutrients optimally.

The average ability of fungal isolates from rumen fluid to degrade hemicellulose is shown in Table 1. The results showed that 5 fungal isolates produced clear zones, so these isolates have the potential to be hemicellulose-producing fungi. This shows a positive reaction to the hemicellulose-degrading enzyme test. Hemicellulose has a very complex structure, consisting of various types of sugars. The largest component of hemicellulose constituent sugars is xylan (Kubata et al., 1994; Saha, 2002). The chemical structure of hemicellulose consists of the main chain (backbone) in the form of D-xylopyranose homopolymers which are connected to each other by  $\beta$ -1,4 D-glycosidic bonds (Saha, 2002) and branch chains in the form of o-acetyl,  $\alpha$ -L-arabinofuranosyl, D-glucuronyl and o-methyl-D-glucuronyl (Ali et al., 2004; Kubata et al., 1994; Saha, 2002). The breakdown of hemicellulose (xylan) involves two enzymes, namely endo- $\beta$ -1,4- xylanase and  $\beta$ -xylosidase.

From the results of several tests carried out on fungal isolates, three isolates were best used as inoculants in fermenting the feed ingredients from agro-industrial waste, namely Cr 1032, Cr 1023 and Cr 1043. The criteria for the best fungal isolates are high ability to degrade lignin and low degradability against cellulose and hemicellulose in feed ingredients. Murni, et al. (2008) stated that the microorganisms that have great ability to decompose lignin but have low degradation of cellulose and hemicellulose are ideal in the bio-conversion of lignocellulose into animal feed.

### Testing of lactic acid bacteria isolated from rumen fluid

The observation showed 11 bacterial isolates from rumen fluid that grew after planting on MRS broth media. The isolates were purified and morphological bacterial colonies were identified. Biochemical tests that took into account the gram of bacteria, tolerance of bacteria

**Table 2.** Results of the morphological identification test of lactic acid bacteria isolates

No.	Code	Morphology of bacteria			
		Form	Elevation	Edge	Color
1	BR1	Cirkular	Raised	Lobed	Yellowish white
2	BR2	Irregular	Raised	Wary	Yellowish white
3	BR3	Irregular	Raised	Wary	Yellowish white
4	BR4	Irregular	Raised	Curly	Yellowish white
5	BR5	Cirkular	Raised	Entire	Yellowish white
6	BR6	Cirkular	Convex	Entire	Milky white
7	BR7	Cirkular	Convex	Entire	Milky white
8	BR8	Cirkular	Convex	Entire	Milky white
9	BR9	Cirkular	Raised	Wary	Yellowish white
10	BR10	Irregular	Flat	Jogged	Yellowish white
11	BR11	Cirkular	Convex	Entire	Milky white

to NaCl levels and at controlled pH and enzyme testing (cellulase, pectinase and chitinase) of bacteria were conducted.

Identification of bacterial colonies was carried out by observing the physical colony of bacteria, paying attention to the shape, color, elevation and edges of the bacterial colony. The results of identification of bacterial colonies can be seen in Table 2.

The results of identification of the bacterial colonies isolated from cattle rumen fluid showed that each bacterial colony had a different shape, elevation, color and edges. The difference in colony morphology indicated that each isolate came from a different species. The morphology of bacterial colonies needs to be observed to facilitate the identification process of bacteria, because the properties of bacterial colonies can determine the type of bacteria (Lay, 1994). From several types of colonies found, it was suspected that all bacterial isolates were lactic acid bacteria, because they could grow on the MRS broth media. The results of observations of isolates that have creamy or milky white color are lactic acid bacteria; this is in accordance with the results of research by Delfahedah et al. (2013) who found several isolates of lactic acid bacteria isolated from sour-sop fruit, namely in the form of round colonies, smooth edges, convex elevation as well as milky white and cream colors.

Bacterial biochemical tests were carried out by observing the gram of bacteria, bacterial tolerance to NaCl levels (4% and 6.5%) and bacterial resistance at a controlled pH (pH 3, pH 4 and pH 5). The results of bacterial biochemical tests can be seen in Table 3.

The results of the gram analysis showed that 11 bacterial isolates from the rumen fluid were gram-positive bacteria, gram-positive bacteria were characterized by no mucus formed by the bacteria after being dripped with KOH, had thick cell walls and thin fat, while gram-negative bacteria had thick fat and cell walls. Thin in the periplasmic space (Kurnia, et al. 2016), KOH will attack the fat (lipid bilayer) and make gram (-) cells burst. The broken cell will release genetic material (DNA) which is an abundant substance in bacterial cells. Very long DNA molecules are sticky strings (resembling mucus, gummy or sticky) which are slimy when removed with an inoculum needle (Edwin, 2011).

The results of the bacterial tolerance test to NaCl levels (4% and 6.5%) showed that the BR7,

**Table 3.** Biochemical test results for bacteria isolated from cattle rumen fluid

No	Code	Testing					
		Gram	NaCl		pH		
			4%	6.5%	3	4	5
1	BR1	+	-	+	+	+	+
2	BR2	+	-	+	-	+	+
3	BR3	+	-	+	-	-	+
4	BR4	+	-	+	-	+	+
5	BR5	+	-	+	-	+	+
6	BR6	+	-	+	+	-	+
7	BR7	+	+	+	+	+	-
8	BR8	+	+	-	+	-	+
9	BR9	+	-	+	-	-	+
10	BR10	+	+	+	-	-	+
11	BR11	+	+	+	+	+	+

Note: - (no reaction); + (reaction)

BR8, BR10 and BR11 bacteria were tolerant to 4% NaCl construction, while in the NaCl concentration of 6.5% there were 10 bacteria that could survive except for BR8 isolates. Salt tolerance test determine the level of resistance of bacterial isolates to bile salts that must be passed in order to reach the intestines and carry out metabolic activity, so as to balance the microflora in digestion (Thakkar et al., 2015).

The results of testing for bacterial resistance at controlled pH (pH 3, pH 4 and pH 5) can be seen in Table 2. Most bacteria can grow at pH 5 conditions and only a few types of bacteria can grow at pH 3 and 4. The lower the pH, the lesser the number of isolates that escape. This is influenced by the lower pH conditions which cause disruption of bacterial activity to grow. The effect of pH on bacterial growth is related to enzyme activity to catalyze the reactions associated with bacterial growth. If the pH of bacterial growth is not optimal, it will disrupt the bacterial growth (Respati, 2017).

The bacterial enzyme test was carried out by growing bacterial isolates on CDA media modified by cellulase, pectinase and chitinase. The isolates that produced a clear zone around the colony indicated that the enzymes suitable for the modified medium were produced. The results of the bacterial enzyme test can be seen in Table 4.

Enzyme-producing bacteria were characterized by the appearance of clear zones on modified CDA media. In the cellulase specification media, there are several bacteria that can produce enzymes, the size of the clear zone indicates that bacteria have different abilities to produce enzymes. This is related to the ability of bacteria to hydrolyze cellulose into glucose, in accordance with the

opinion of Rahayu, et al. (2014) who stated that the bacterial isolates that have high cellulase enzyme activity can hydrolyze cellulose to glucose and show a large clear zone around the colony, due to the changes in the structure of cellulose from fibrous to glucose with the structure being non-fiber.

The pectinase activity of bacterial isolates can be seen in Table 3. The ability of bacteria to produce clear zones on pectinase-specific media identifies that these bacteria have the ability to produce the pectinase enzyme. The larger the clear zone produced by the bacterial isolate, the greater the ability of the bacteria to produce the pectinase enzyme. In the test of chitinase-producing bacteria, there are several bacteria that can produce clear zones. The clear zone is formed due to the process of breaking the chitin substrate into simple compounds after chitinase is secreted by bacteria (Suryadi et al., 2013).

The results of the selection from tests carried out on bacterial isolates from rumen fluids as inoculants in the fermentation of feed ingredients obtained the five best isolates, namely BR6, BR7, BR8, BR10 and BR11. The addition of lactic acid bacteria as an inoculant in fermentation is used to prevent the growth of destructive bacteria during the fermentation process. Lactic acid bacteria can produce nisin as bacteriocin, which is a biopreservative compound that can prevent the growth of destructive bacteria or even pathogenic bacteria (Ray, 1992).

## CONCLUSIONS

There are three fungal isolates that can be used as inoculants in fermenting feed ingredients from waste, namely Cr 10<sup>3</sup>2, Cr 10<sup>2</sup>3 and Cr 10<sup>4</sup>3, the three isolates show great ability in degrading lignin. The five best isolates of lactic acid bacteria were obtained, which had quite a wide ability as a preservative in the fermentation of feed ingredients from agro-industrial waste, namely BR6, BR7, BR8, BR10 and BR11.

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**Table 4.** Diameter of bacterial growth on enzyme tests (cellulase, pectinase and chitinase)

No	Bakteri Code	Enzyme		
		Selulase	Pectinase	Kitinase
1	BR1	-	-	-
2	BR2	10 mm	3 mm	12 mm
3	BR3	12 mm	6 mm	11 mm
4	BR4	9 mm	4 mm	6 mm
5	BR5	10 mm	3 mm	5 mm
6	BR6	10 mm	4 mm	5 mm
7	BR7	-	-	6 mm
8	BR8	5 mm	-	-
9	BR9	9 mm	6 mm	5 mm
10	BR10	9 mm	-	13 mm
11	BR11	-	-	5 mm

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