POTENCY OF LIGNOCELLULOSE DEGRADING BACTERIA ISOLATED FROM BUFFALO AND HORSE GASTROINTESTINAL TRACT AND ELEPHANT DUNG FOR FEED FIBER DEGRADATION

A. Wahyudi[1], M. N. Cahyanto[2], M. Soejono[3] and Z. Bachruddin[3]

[1]Department of Animal Production , Faculty of Agriculture and Animal Science, Muhammadiyah University of Malang, Jl Raya Tlogomas 246 Malang 65144 - Indonesia

[2]Faculty of Agriculture Technology University of Gadjah Mada, Yogyakarta - Indonesia

[3]Faculty of Animal Science and Industry University of Gadjah Mada, Yogyakarta - Indonesia

Corresponding E-mail: wahyudi_biotek@yahoo.co.id

Received November 14, 2009; Accepted February 12, 2010

ABSTRACT

Lignin is limiting factor for cellulose and hemicellulose degradation in rumen. Isolation and selection bacteria from buffalo and horse gastrointestinal tract and elephant dung could be found bacteria that have superiority to degrade lignin, xylan, and cellulose. Those animals were chosen because they were herbivores that consume low quality crude fiber as their main energy sources. Lignocellulose degrading bacteria were isolated by Hungate selective media, by using lignin (tannic acid), xylan, and cellulose as selective substrates. The morphological identification used an enrichment media by measuring color, colony size, diffusion zone, clear zone, and biochemical identification using production of ligninase, xylanase, and cellulase enzymes. The best lignocellulose degrading bacteria then was determined by the morphological and biochemical character. This study showed that lignocellulose degrading bacteria could be found in gastrointestinal tract of buffalo and horse, and elephant dung. Highest number colony was found in samples from buffalo's colon (376), followed by horse's cecum (203), elephant's dung (46), buffalo's cecum (23), buffalo's rumen (9) and horse's colon (7). The highest isolates activity of lignolytic, xylanolytic, and cellulolytic were reached by buffalo's cecum (7.64), horse's cecum (6.27), and buffalo's colon (2.48). Meanwhile the highest enzymes productivities were: buffalo's cecum (0.0400 µmol), horse's cecum (1.3912 µmol) and buffalo's colon (0.1971 µmol). Based on morphologycal character and biochemical test, it could be concluded that lignolytic from buffalo's cecum, xylanolytic from horse's cecum, and cellulolytic from buffalo's colon were the superior isolates and they were 99% analyzed as Enterococcus casseliflavus/gallinarum species.

Keywords : bacteria, buffalo, degradation, elephant, feed fiber, horse, lignocellulose.

INTRODUCTION

Lignocellulose degrading bacteria has important role in energy supply for ruminants. Ruminants are able to convert low quality feed in rumen because role of the lignocellulolytic bacteria. Tropical buffalo can grow properly with low quality roughage, agricultural and industrial waste with basic structure high lignocellulose as main energy source (Wanapat, 2003). On the other hand, conversion of feed crude fiber to animal product was not optimum in intensive animal farming (especially dairy cattle). Only 10-35% energy from crude fiber can be used, while 20-70% undigested cellulose were carried out with feces (Varga dan Kovler, 1997). These were the prove that crude fiber degradation in rumen was not optimal and these crude fiber content in feces was still high and fermentable (Krause *et al*., 2003).

Lignocellulolytic bacteria existence in the rumen is influenced by feed, chemical substance, and environment stress factor. Using too much concentrate and chemical substance in intensive farming system would depress rumen lignocellulolytic bacteria population. In contrast, these microbes could grow rapidly in ruminants fed roughage, so that herbivores that consume

high crude fiber would be good to be used as lignocellulolytic microbes source. The microbes perhaps could be found in cecum and colon due to their colonized with undigested crude fiber in rumen.

Consideration of low crude fiber degradation in dairy cattle, and potentiality of lignocellulose degrading microbes from cecum and colon herbivores, lead to conduct isolation for collecting lignocellulolytic bacteria that has high ability to improve crude fiber degradation.

Cellulolytic bacteria has been isolated often to get anaerobic bacteria that degrade cellulose and hemicellulose. Study of enzymes capability and fermentation media formulation also has been conducted oftenly. Isolation of lignin degrading bacteria (Martani, 2003) and lignocellulolytic fungi (Samingan, 1998) has been done, but both have been done in aerobic isolation.

Lignin degrading bacteria is presumed live both in rumen and colon, but has not been proven empirically. Further exploration about potency of lignocellulose degrading bacteria from cecum and colon, so far is limited. Lignocellulolytic from cecum and colon could be has better ability to degrade crude fiber because substrate in those location contain rumen undigested crude fiber. The coprophagy phenomenon in rabbit, and elephant fed their feces for the calf showed the more perfection process of feed fermentation and nutrient fiber usage by lignocellulolytic by non ruminants herbivore.

Besides as differ factor from the previous study, isolation of lignocellulose degrading bacteria from buffalo's cecum and colon, exploration of lignocellulose degrading bacteria from horse's cecum and colon and also elephant dung are the new issue added in this study. Isolation from various compartment from herbivores are expected for getting potential bacteria for probiotics or inoculum for fiber feed anaerobic degradation.

This study was conducted to isolate and identify lignocellulose degrading bacteria from herbivore's digestive tract anaerobically and then test their ability in synthetic medium to degrade feed fiber.

MATERIAL AND METHODS

Isolate Sources

The bacteria were isolated from fresh sample of buffalo's rumen, cecum and colon, horse's' cecum and colon, and elephant's dung. Buffalo's sample were taken from Demak' slaughter house (abattoir), horse's sample from Imogiri, and Elephant's dung from Gembiraloka Zoo, Yogyakarta.

Solid Media and Isolation

Microbes from all samples were grown in solid media by Hungate method (Ogimoto and Imai, 1981): weigh 0,02g KH_2PO_4; 0,03g K_2HPO_4; 0,01g $MgSO_4$; 0,01g $CaCl_2$; 0,10g NaCl; 0,10g $(NH_4)_2SO_4$; 0,10ml Rezasurin 0,1% solution; 0,02g Cystein-HCl.H_2O; 0,40g Na_2CO_3; 30,00ml rumen liquid; 1,00g substrate; 70,00ml Aquadest and 1,8% Agar. Selective substrate used were lignin, xylan and cellulose. All ingredients were mixed in Erlenmeyer (except substrate that were sterilized by 5 ml aquadest in tube), pH was determined 6,8 and heated until all ingredients dissolved. The flask then transferred aseptically with oxygen-free CO_2 gas displacing all air until red color faded, closed with rubber stopper, sealed, then sterilized with its content in 12 psi for 20 minutes. In warm condition, media was divided into 3 tubes. Each selective substrate then dissolved, then poured 4,5 ml each into 5 mm petri disc. Microbes source liquid (50µl) with 10^{-5} dilution then were inoculated for 7-14 days in anaerobic jar that filled by anaerobic generating kit. The growing colonies then were counted and identified.

Qualitative Selection

The lignin degrader bacteria was selected qualitatively based on the diffusion zone diameter that formed around colony (Subbarao, 1993: Samingan, 1998: Martani, 2003). While xylan and cellulose degrading bacteria were selected by measured clear zone around colony (Ogimoto and Imai, 1981). Each isolate was inoculated by spot method on nutrient agar that contain 1% tannic acid (Subbarao, 1993). Cellulose and xylan degrader were isolated according clear zone around colonies on nutrient agar that contain 1% cellulose and 1% xylan respectively (modified Hungate method in Ogimoto and Imai, 1981). Diffusion and clear zone were measured after 7 days of anaerobic incubation. Ratio between diffusion or clear zone with colony size was used to determine the selected isolates.

Liquid Media

Isolates were grown in liquid media by

modified Hungate method (Bachruddin, 1985) which were mixed 150 ml mineral I solution, 150 ml mineral II solution, 1 ml rezasurin 0,1% (w/v) , 2,00g substrate, 400 ml rumen liquid extract, 2 g yeast extract as enrichment nutrient, and 250 ml aquadest in 1000 ml Erlenmeyer. Substrates that used were mixed lignin, xylan and cellulose, adjusted by each enzymes production test. All materials in Erlenmeyer then were heated 100^{o}C for 5 minutes for homogenized along with CO_2 gas. Temperature was sustained 45^{o}C in water bath. An aerobic condition was reached when red color was faded. Then, 32,3 ml sodium carbonate and 16,7 ml Cystein-HCl were added. Tube then was closed by rubber stopper and sealed then sterilized in 121^{o}C for 15 minutes. Each media (according to selective substrate) was divided for isolates number that would be grown in 50 ml serum bottle. Isolate from solid media was dissolved in dilute solution in 0,5 λ 600 absorbent, inoculated in bottle as much as 10%, incubated in 39^{o}C for 7 days. Growth culture media then was used as enzymes source.

Quantitative Selection

Enzyme extract was collected from centrifuged liquid media culture in 12.000 x g for 15 minutes in 4^{o}C. According to the substrate, extracts were tested in three kinds of substrates that contained 1% Whatman No.1 filter paper/xylan/lignin in 50 mM acetate buffer and pH 5,5. Each substrate liquid in buffer was taken 8 ml, added 1 ml enzymes source, and 1 ml aquadest. The mixture then were shaken by fortex, enzyme activity was measured in 60 minutes. Reduction sugar (glucose from filter paper, xylose from xylan), or vanillin from lignin produced from the reaction were the enzyme activities (Efiok, 1996). For sugar reduction: 1 ml of sample was added to 3 ml DNS reagent and 1 ml aquadest (Miller, 1959), for vanilin: 1 ml of sample was added to 4 ml methanol, then measured the absorbent by spectrophotometer in λ 560 nm for glucose, 550 nm for xilosa and 335 nm for vanilin.

Research Design

The research was conducted based on qualitative and quantitative analysis. One way completely randomized design was used as statistically design. Six isolates sources were used as treatment with three replication and filter paperase, xylanase, and ligninase as parameters observed.

RESULT AND DISCUSSION

Isolation of Lignocellulolytic Bacteria

Samples were taken from lignocellulose material of buffalo's and horse's digestive compartment and also from elephant's dung. The compartments were consisted of buffalo's rumen, cecum and colon, horse's cecum and colon and elephant's dung (Table 1). Because of using lignin (tannic acid), xylan and cellulose as selected substrate in bacteria isolated media, the cultures were predicted containing lignolytic, xylanolytic and cellulolytic bacteria respectively. Every strain of bacteria needed a specific substrate as an energy source (Berra-Maillet *et al*., 2004).

Lignin, xylan, and cellulose degrading bacteria could be found from all samples. In 10^{-5} dilution, the highest number of lignin degrading isolates was found from buffalo's colon (3), horse's cecum for xylan (188), and also buffalo's colon for cellulose (262). Buffalo's colon seem contain more lignocellulolytic bacteria than others. Puppo *et al*. (2002) and Wanapat (2003), stated that buffalo has higher fiber degrading bacteria than others animal farming, especially local cow.

Herbivores digestive tract, specifically ruminants in Indonesia, in general contain high lignocellulose feed, so that lignocellulose degrading bacteria was expected exist. This study proved that all compartment digestive tract of buffalo and horse, and also elephant dung possess lignin, xylan, and cellulose degrading bacteria. Several previous study showed that lignocellulose degrading bacteria was exist in rumen and goat's feces has used as rumen microbes replacement (Utomo *et al*., 2006). Another study showed that horse's cecum and colon, and elephant have microbes composition such as rumen. (Ulrey *et al*., 1997). According to Table 1, it is proved that the lignocellulolytic microbes could be found from herbivores digestive tract.

Qualitative of Lignocellulolytic Activity

Qualitative selection (Table 2) showed that isolates from buffalo's cecum has highest activity in degrade lignin (7,64) followed by colon (3.98) and rumen (3.85) These data proved that lignolytic bacteria from buffalo has higher lignin degradation than others. This statement is strengthen by Wanapat (2003), Kennedy *et al*. (1992) and Hardjosubroto (2006) studies that

Table 1. Number of Lignocellulolytic Colonies in 10^{-5} Dilution

No	Isolate Source	Code	Colonies (cfu)			Total of colonies (cfu)
			Lignin degrader	Xylan degrader	Cellulose degrader	
1	Buffalo's rumen	Rkb	1	4	4	9
2	Buffalo's cecum	Skb	2	10	11	23
3	Buffalo's colon	Kkb	**3**	111	**262**	376
4	Horse's cecum	Skd	1	**188**	14	203
5	Horse's colon	Kkd	spread	3	4	7
6	Elephant's dung	Fg	2	12	32	46

Rkb=Buffalo's rumen, Skb=Buffalo's cecum, Kkb=Buffalo's colon, Skd=Horse's cecum, Kkd=Horse's colon, Fg=Elephant's dung

buffalo could use fiber feed more efficient than other ruminants. This statement also support the hypothesis that fiber degrader bacteria from lower tract has higher ability than those from rumen.

The highest activity of xylan degrader isolate (Table 3) found from horse's cecum (6.27) and followed by buffalo's cecum (3.32) and then rumen (2.84) It still unclear explanation why horse bacteria has higher ability than others, even though buffalo's colon has also high number of this bacteria (Table 1)

Xylan is main carbohydrate that form hemicellulose, consist of xylosa polymer and other sugar with β-1,4, bond and end side chain with α-1,2 or α-1,3 bonds (Peres *et al.*, 2002). Differences of heteropolymerisity of sugar that form xylan in rumen and cecum or colon in this study are predicted as main reason why rumen and cecum xylanolytic bacteria have higher activity than colon xylanolytic bacteria .

Cellulolytic isolate from buffalo's colon has the highest activity (2.48) in cellulose degradation than others (Table 4). Buffalo's colon isolate seem has higher activity than isolate from horse's digestive tract and elephant's dung. This data strengthen the Puppo's *et al*. (2002) and Wanapat's (2003) statement that buffalo has higher fiber degrading bacteria than others animal farming. From buffalo point of view itself, cellulolytic isolate from colon is superior than bacteria from rumen (1,92) or cecum (1,40). This study also prove the hypothesis that lignocellulolytic from colon has higher activity in fiber degradation.

Quantitative Lignocellulolytic Activity

Several lignin, xylan, and cellulose degrading isolate were superior than the others (Table 5). It was concluded from quantitative analysis of enzymes productivity. The lignolytic isolates was from buffalo's cecum, xylanolytic was from horse's cecum, and cellulolytic was from buffalo's colon with enzyme productivities were 0.0400 µmol, 1.3912 µmol and 0.1971 µmol respectively. The data showed positive correlation with qualitative analysis based on each colonies activity ratio. Qualitatively, buffalo's cecum, horse's cecum and buffalo's colon were best selected among others isolates, with activity ratio 7,64; 6,27 and 2,48 (Table 2, 3 and 4).

Quantitative and qualitative test for ligninase, xylanase and cellulase enzymes activities showed that selected lignocellulolytic bacteria was found more frequently from cecum and colon both from buffalo and horse. Its strengthen the hypothesis above that lignocellulose degrading bacteria could be found from lower digestive tract of herbivores, including ruminants. This statement support Ullrey *et al*. (1997) study that cecum and colon monogastric herbivores have similar composition microbes with ruminants. Futhermore this study showed that the cecum and colon lignocellulolytic bacteria have higher ability to degrade lignocellulose than rumen bacteria.

The three selected bacteria then were tested for classification up to species. Result of morphological character and biochemical test were summarized in Table 6. Lignolytic isolate from bufallo's cecum, xylanolytic from horse's cecum and cellulolytic from horse's colon have a similarity. According to microscopic, macroscopic, and biochemical analysis by Bergey's manual determinative bacteriology (Holt *et al*., 1994) and Atlas of Rumen Microbiology

Table 2. Lignolytic Activities

Isolate code	Colony's color	Diameter of colony (mm)	Diameter of diffusion zone (mm)	Activity ratio
Rkb	white-cream	4.4	16.93	3.85
Skb	white-cream	2.17	16.57	**7.64**
Kkb	white-cream	5.27	20.97	3.98
Skd	white-cream	4.35	16.5	3.67
Kkd	white-cream	9.2	-	-
Fg	white-cream	9.21	-	-

Table 3. Xylanolytic Activities

Isolate code	Colony's color	Diameter of colony (mm)	Diameter of clear zone (mm)	Activity ratio
Rkb	white-cream	4.35	12.35	2.84
Skb	white-cream	4.6	15.25	3.32
Kkb	white-cream	4.83	5.8	1.2
Skd	white-cream	3	18.8	**6.27**
Kkd	white-cream	4.18	-	-
Fg	white-cream	5.42	14.27	2.63

Table 4. Cellulolytic Activities

Isolate code	Colony's color	Diameter of colony (mm)	Diameter of clear zone (mm)	Activity ratio
Rkb	white-cream	2.75	3.85	1.4
Skb	white-cream	10.07	19.33	1.92
Kkb	white-cream	7.85	19.5	**2.48**
Skd	white-cream	10.23	11	1.08
Kkd	white-cream	5.13	-	-
Fg	white-cream	2.97	6.6	2,22

Table 5. Lignocellulolityc Enzyme Productivities

Isolate code	Ligninase (µmol)	Xylanase (µmol)	Cellulase (µmol)
Rkb	0.02	0.3	0.13
Skb	**0.04**	0.34	0.06
Kkb	0.03	0.29	**0.2**
Skd	0.01	**1.39**	0.11
Kkd	0.02	0.38	0.2
Fg	0.02	0.37	0.11

(Ogimoto and Imai, 1981), the three isolates tend to be *Ruminococcus sp* genus, but based on biochemical analysis by BD Phoenix automated microbiology system for identification with 99% confidence value, the isolates were *Enterococcus casseliflavus/gallinarum*.

CONCLUSION

Lignocellulolytic isolates could be found from all samples in this study. Based on morphologycal character and biochemical test, it could be concluded that lignolytic isolate from buffalo's cecum, xylanolytic from horse's cecum, and cellulolytic from horse's colon were the superior isolates. The classification of the three isolates were Enterococcus casseliflavus/ gallinarum. High ability of lignocellulose degrading bacteria that have isolated need formulated and study further to find out an optimum fermentation inoculum for feed fiber degradation.

Table 6. Characteristic of Lignocellulolytic Isolates

Parameter	Lignolytic isolate from buffalo's cecum	Xylanolytic isolate from horse's cecum	Cellulolytic isolate from buffalo's colon
Colony Morphology			
Color	white-cream	white-cream	white-cream
Shape	Circular	Circular	Circular
Border	Entire	Entire	Entire
Elevation	Raised	Raised	Convex
Inner structure	Translucent	Translucent	Translucent
Cell Morphology			
Shape	Coccus (single/double)	Coccus (single/double)	Coccus (single/double)
Size (µm)	0.93	1.12	1.2
Endospore	-	-	-
Gram	+	+	+
Motility	-	-	-
Biochemical test			
Arginine-Arginine-Amc	-	-	-
L-Arginine-Amc	-	-	-
L-Leucine-Amc	+	+	+
L-Pyroglutamic Acid-Amc	+	-	+
3-Methyl Glutaric Acid	-	-	-
D-Gluconic Acid	+	+	+
Alpha Keto Glutaric Acid	+	+	+
Thymidine	+	+	+
4Mu-Bd-Galactoside	+	+	+
4Mu-N-Acetyl-Bd-Glucosaminide	+	+	+
Alanine-Alanine-Pna	-	-	-
Pnp-Ad-Glukoside	+	+	+
Dextrose	+	+	+
D-Trehalose	+	+	+
N-Acetyl-Glucosamine	+	+	+
Glycine-Proline-Amc	-	-	-
L-Hystidine-Amc	-	-	-
L-Phenylalanine-Amc	+	+	+
L-Tryptophan-Amc	+	+	+
Colistin	+	+	+
D-Mannitol	+	+	+
3-Methyladipic Acid	-	-	-
4Mu-Ad-Glucoside	+	+	+
4Mu-Bd-Glucoronide	-	-	-
4Mu-Phosphate	-	-	-
L-Proline-Pna	-	-	-

(continued on the next page)

Table 6. Characteristic of Lignocellulolytic Isolates (continued)

Parameter	Lignolytic isolate from buffalo's cecum	Xylanolytic isolate from horse's cecum	Cellulolytic isolate from buffalo's colon
Pnp-Phosphate	-	-	-
D-Sucrose	+	+	+
Maltose	+	+	+
Urea	-	-	-
L-Alanine-Amc	-	-	-
L-Isoleucine-Amc	-	-	-
L-Proline-Amc	-	-	-
Methionine-Amc	-	-	-
D-Fructose	+	+	+
Iminodiacetic Acid	-	-	-
Polymyxin-B	+	+	+
4Mu-Bd-Cellobioside	+	+	+
4Mu-Bd-Glucoside	+	+	+
4Mu-Phosphate (with Trehalose)	-	-	-
Valine-Alanine-Pna	-	-	-
Beta-Gentibiose	+	+	+
D-Tagatose	-	-	-
Maltotriose	+	+	+
Esculin	+	+	+
99% Confidence value of species prediction	*Enterococcus casseliflavus/ gallinarum*	*Enterococcus casseliflavus/ gallinarum*	*Enterococcus casseliflavus/ gallinarum*

ACNOWLEDGEMENTS

This publication is part of Doctoral Grant Research that funding by Institute for Research and Community Services of Gadjah Mada University.

REFFERENCES

Bachruddin, Z. 1985. Development of Ruminal Microflora in Goat (*Capra hircus*). Thesis. Graduate School, University of Philipines, Los Banos.

Berra-Maillet, C., Y. Ribot and E. Forano. 2004. Fiber-degrading system of different strains of the genus fibrobacter, Appl. Environ. Microbiol. Apr: p. 2172-2179

Efiok, B.J.S. 1996. Basic Calculation for Chemical and Biological Analysis. AOAC International, Maryland, USA.

Hardjosubroto, W. 2006. Kerbau, mutiara yang terlupakan. Prosiding Orasi dan Seminar Pelepasan Dosen Purna Tugas 2006, Menyongsong Rencana Kecukupan Daging Tahun 2010, Fakultas Peternakan UGM. Hal. 56-102

Holt, J.G, N.R Krieg, P.H.A Sneath and S.T. William. 1994. Bergey's Manual of Determinative Bacteriology. 9th Ed., Willian & Wilkins, USA.

Kennedy, P.M., C.S. McSweeney, Foulkes, A. John, A.C. Schlinka, R.P. Lefeuvre, and J.D. Kerr. 1992. Intake and digestion in swamp buffaloes and cattle : The digestion of rice straw (*Oriza sativa*), J. Agric. Sci., 119 (2) : 227-242

Krause, D.O., S.E. Denman, R.I. Mackie, M. Morrison, A.L. Rae, G.T. Attwood, and C.S. McSweeney. 2003. Opportunities to improve fiber degradation in rumen: microbiology, ecology and genomics, FEMS Microbiol. Rev. 27: 663-669

Martani, E., N. Haedar and S. Margino, 2003. Isolation and characterization of lignin degrading bacteria from several natural

substrate. Gama Sains V (2) : 32 – 35.

Ogimoto, K. and S. Imai. 1981. Atlas of Rumen Microbiology. Japan Scientific Societies Press, Tokyo.

Peres, J., J. Munoz-Dorado, T. de la Rubia, and J. Martinez. 2002. Biodegradation and biological treatment of cellulose, hemicellulose and lignin: an overview. Int. Microbiol. 5 : 53-56.

Puppo, S., S. Bartocci, S. Terramoccia, F. Grandoni, and A. Amici. 2002. Rumen microbial counts and *in vivo* digestibility in buffaloes and cattle given different diets. J. Anim. Sci. 75: 323-329.

Samingan. 1998. Biodegradation of Acacia mangium Wild offal by lignocellulolytic fungi. Thesis, Graduate School, University of Gadjah Mada, Yogyakarta.

Subbarao, N.S. 1993. Biofertilizers in Agriculture and Forestry. 3rd ed. International Science Publisher, New York.

Ullrey, D.E, S.D Crissey and H.F. Hintz. 1997. Elephants : Nutrition and Dietary Husbandry. Michigan State University. East Lansing, Michigan 48824

Utomo, R., Z. Bachruddin, B.P. Widyobroto, M. Soejono and R. Antari. 2006. The use of Goat' feces as the microbial resources and microbial enzyme as a substitute for rumen liquor. Bulletin Peternakan. 30 (3):106-114

Varga, G.A. and E.S. Kovler. 1997. Microbial and animal limitation to fiber digestion and utilization. J. Nutr. 127 (5) :819-823

Wanapat, M. 2003. Comparative study between swamp buffalo and native cattle in feed digestibility and potential transfer of buffalo rumen digesta into cattle. Asian-Aust. J. Anim. Sci. 16 (4):504-510