Effect of Administration of Lignocellulose-Degrading Fungi Isolated from Herbivore's Gastrointestinal Tract for Fiber Degradation

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Abstract. Isolation and selection of lignocellulose-degrading fungi from compartment of herbivore's gastrointestinal tract were predicted found fungi that have superiority to degrade lignin, xylan, and cellulose. Lignocellulose-degrading fungi were isolated from compartment of buffalo's and horse's gastrointestinal tract and also elephant dung with malt extract agar, using cellulose, xylan, and tannic acid as selective substrate. Morphological and biochemical test had been done to get superior isolates. This study showed that lignocellulose-degrading fungi could be found in all sample of buffalo's and horse's gastrointestinal tract and also elephant dung. The highest number of lignin, xylan and cellulose-degrading isolates respectively were found from buffalo's cecum (5 isolates), buffalo's colon (19 isolates), and buffalo's colon (326 isolates). The highest isolates activity of lignolytic, xylanolytic, and cellulolytic respectively were reached from horse's cecum (2.38), horse's cecum (6.67), and buffalo's colon (5.60). Meanwhile the highest enzymes activities were reached from horse's cecum (0.166 Unit/g protein), horse's cecum (5.037 Unit/g protein) and buffalo's colon (2.488 Unit/g protein). From this study could be concluded that lignocellulose-degrading fungi could be found from all compartment of herbivore's gastrointestinal tract. Based on quantitative and qualitative selection, lignolytic from horse's cecum, xylanolytic from horse's cecum and cellulolytic from buffalo's colon were superior isolates and predicted as *Aspergillus sp* genus. Administratiton of *Aspergilillus sp* inoculum into rumen fluid medium elevated significantly dry matter, organic matter, crude fiber, neutral detergent fiber, and acid detergent fiber digestibility as 4.55%; 4.45%; 6.69%; 11.65% and 8.23% respectively.

Key Words : lignocellulose-degrading fungi, herbivore's gastrointestinal tract, fiber degradation.

Introduction

Anaerobic fungi are unique among herbivore's gastrointestinal tract microorganisms in that they able penetrate cuticle of plant tissue (Gordon and Phillips, 1998). Residues after incubation with fungi are physically weaker than those incubated with rumen bacteria or rumen fluid. Rumen fungi produce high level of cellulases and xylanases, but these enzyme are regulated by soluble sugar available to the organisms. Existence of rumen fungi is influenced by feed, chemical substance, and environment stress condition. Using too much concentrate and chemical substance in intensive farming system would depress rumen's fibrolytic population. In contrast, these microbes could grow properly in ruminants that fed by roughage. Herbivores that consume high level of crude fiber would good to be used as lignocellulose-degrading microbes source including fungi. These fungi perhaps could be found in all compartment of herbivore's digestive tract, especially in lower gut such as cecum and colon due to the colonized undigested crude fiber in rumen.

Anaerobic fungi have been found in the gut of ruminants such as buffalo, sheep and cattle, including deer and impalas (Gordon an Phillips, 1998). Further, similar fungi are known present in the horse cecum and stomachs of marsupials, including kangaroos and wallabies. These types of anaerobic fungi also have been culture from the elephant's dung. The fungi were predicted particularly active as fiber-degrader. A common characteristic of rumen fungi related to ruminant nutrition is their ability to colonize extensively the lignin-containing plant cell wall of forages.

Buffalo has ability used feed fiber more efficient than beef cattle (Wanapat, 2001; Hardjosubroto, 2006). Cellulose could be degraded twice more effective in buffalo's rumen than in beef cattle's rumen, and enzyme activity of buffalo's rumen fluid was higher than another rumen's fluid. Buffalo also has fungi population twice more than beef cattle (Puppo et al., 2002; Wanapat, 2001). Consideration of potentiality of lignocellulose-degrading fungi in buffalo, isolation is important to be done to get lignocellulose-degrading fungi 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 media formulation also has conducted often. Isolation of lignin degrading bacteria (Martani et al., 2003) and lignocellulolytic fungi (Samingan, 1998) has been done, but both of them have been done in aerobic isolation. Lignin degrading fungi is presumed live both in rumen and colon, but has not been proven empirically. Further exploration about potency of lignocellulose-degrading fungi form cecum and colon, so far is limited. Lignocellulose-degrading fungi from cecum and colon could be has better ability to degrade crude fiber because substrate in those location contain rumen-undigested crude fiber.

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

Materials and Methods

Isolate Sources

The fungi 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.

Solid Media and Isolation

Microbes from all sample were grown in solid media by modified of Subbarao methods (Martani et al., 2003). 0.5% (w/v) tannic acid, xylan, or cellulose was used as selective substrate in 1.5% (w/v) malt exstract agar (MEA) medium in different flask for degrading lignin, xylan and cellulose isolate respectively. 0.10 ml rezasurin 0.1% solution was used as anaerobic indicator. The flask then transferred aseptically with oxygen-free CO_2 gas displacing all air until red indicator faded, closed with rubber stopper, sealed, then sterilized with its content in 12 psi for 20 minutes. Media poured 4,5 ml each into 5 mm petri disc on warm condition. Microbes source liquid (50µl) with 10^{-3} dilution then inoculated for 5 days in *anaerobic jar* that filled with *anaerobic generating kit*. The growing colonies then counted and identified.

Qualitative Selection

The lignin degrading fungi was selected qualitatively based on the diffusion zone diameter that formed around colony using Subbarao methods (Martani et al., 2003). Each lignolytic isolate inoculated with spot method on nutrient agar that contain 1% tannic acid. Xylan and Cellulose degrading fungi were isolated according to clear zone around colonies on nutrient agar that contain 1% cellulose and 1% xylan respectively. Diffusion and clear zone were measured on 5 days anaerobic incubation. Ratio between diffusion or clear zone with colony size was used to determine the selected isolates.

Liquid Media

Isolates were grown in growth media contain 1.5% (w/v) malt extract/ME using Subbarao methods (Martani et al., 2003) by added 1% B complex vitamins as enrichment nutrient and 0.10ml Rezasurin 0,1% solution as anaerobic indicator in a flask. The flask then transferred aseptically with oxygen-free CO_2 gas displacing all air until red indicator faded, losed with rubber stopper, sealed, then sterilized with its content in 12 psi for 20 minutes. Each media (according selective substrate) divided for isolates number that

would be grown in 50 ml serum bottle. Spore from solid media dissolved in dilute solution on 7.5 x 10^5 spore per ml using haemocytometer and inoculated in bottle as much as 10%, incubated in 39^{o}C for 5 days growth culture media then used as enzyme source.

Quantitative Selection

Enzyme extract was obtained 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 kind of substrates that contain 1% lignin/xylan/Whatman No.1 filter paper 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 shake with fortex, enzyme activities were measured in 60 minutes. Vanillin from lignin and reduction sugar (xylose from xylan, glucose from filter paper) that produce from the reaction was the enzyme activity (Efiok, 1996). The product tested with Miller methods, for sugar reduction: 1 ml sample added to 3 ml DNS reagent and 1 ml aquadest, for vanillin: 1 ml sample added to 4 ml methanol, then measured the absorbent with spectrophotometer in λ 560 nm for glucose, 550 nm for xylose and 335 nm for vanillin.

Inoculum's Implementation for Rice Straw Degradation

Selected isolates was used as inoculum in Tilley and Terry's in vitro culturing methods. Each liter of McDougall's buffer contain 9.8 g $NaHCO_3$, 7.0 g $Na_2HPO_4.7H_2O$, 0.57 g KCl, 0.47 g NaCl, 0.12 g $MgSO_4.7H_2O$ and 1 ml $CaCl_2$ 4% solution. In warm condition (39^{o}C) it's diluted and homogenized along with oxygen-free CO_2 gas at pH 6.8. Then fill 0.5 g rice straw, 7 ml rumen fluid (substitute 10% with inoculum contain 7.5 x 10^2 spores/µl) and 28 ml Mc Dougall's buffer in serum flask with oxygen-free CO_2 gas displacing all air. Close with rubber stopper and incubated in 39^{o}C for 48 hours then measured dry matter (DM), organic matter (OM), crude fiber(CF), neutral detergent fiber(NDF) and acid detergent fiber(ADF).

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 and selected isolates were used as treatment. Ligninases, xylanases, filter paperases, DM, OM, CF, /NDF and ADF were measured as the parameters.

Results and Discussion

Isolation of Lignocellulose-degrading Fungi

Samples were taken from lignocellulose material of buffalo's and horse's digestive compartment and also from elephant's dung, so there were six compartments. The compartments were buffalo's rumen, cecum and colon, horse's cecum and colon and elephant's dung respectively (Table 1). Because of using lignin, xylan and cellulose in isolated media as a selected substrate, the cultures were predicted contain lignin, xylan and cellulose degrading fungi respectively. Every isolate of fungi needed a specific substrate as an energy source (Berra-Maillet et al., 2004).

This study showed that lignocellulose degrading fungi could be found in all gastrointestinal tract compartment of buffalo and horse, and also elephant's dung. Lignin, xylan, and cellulose degrading fungi could be found from all samples. Oleyeke and Okusanmi (2008) showed that the composition of fungi isolated from rumen were *Mucor* (40.6%), *Penicillium* (23.4%) and *Aspergillus* (14.7%). Base on the place, the total colony number was found from buffalo's' colon (363), then followed by bufallo's rumen (283), horse's cecum (272), buffalo's cecum (246), elephant's dung (162) and horse's colon (148). But, in 10^{-3} dilution, the highest number of lignin degrading isolates found from buffalo's cecum (5 isolates), buffalo's colon for xylan (19 isolates), and buffalo's colon for cellulose (326 isolates). Buffalo's colon seem contain more lignocellulose degrading fungi than others. Puppo et al. (2002) and Wanapat (2001), stated that buffalo has higher fiber degrading fungi than others animal farming, especially local cows.

Herbivores digestive tract, specifically ruminants in Indonesia, in general fed with feed contain high lignocellulose, so that lignocellulose degrading fungi was expected

exist. This study proved that all compartment digestive tract of buffalo and horse, and also elephant's dung possess lignin, xylan, and cellulose degrading fungi. Several previous study showed that lignocellulose degrading fungi 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 also elephant's cecum and colon have microbes composition such as rumen (Ulrey et al*.,* 1997). According Table 1, it is proved that the lignocellulose degrading fungi could be found from herbivores digestive tract.

Qualitative Analysis

Qualitative selection (Table 2) showed that isolates from horse's cecum has highest activity ratio in lignin degradation (2,38) followed by buffalo's colon (2.35) and buffalo's rumen (2.31), but these differences were not significant. Li et al. (2007) showed that anaerobic fungi isolated from herbivore's digestive tract secrete highly active lignocellulose-degrading enzyme. These data proved that lignolytic fungi from horse's cecum and buffalo's colon have similarity with rumen fungi. This statement is strengthen by Ulrey et al. (1997) that horse's cecum and colon have microbes composition such as rumen. This statement also support the hypothesis that fiber degrader fungi from lower tract has tended better ability than rumen. Furthermore Paul et al. (2004) showed that anaerobic fungus isolated from wild ruminant's feces could increase activity of cellulases and xylanases.

Highest activity ratio of xylan degrading fungi (Table 3) has found from horse's cecum (6.67) and followed by buffalo's cecum (6.05) and then elephant's dung (5.89). It still unclear explanation why horse's cecum fungi has better ability than others, even though buffalo's colon has high number of this fungi. Xylan is main carbohydrate that in hemicellulose formation consist of xylosa polymers 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 forming xylan in cecum, rumen or colon in this study are predicted as main reason why cecum's xylanolytic fungi have higher activity than colon.

Cellulolytic fungi from buffalo's colon has highest activity (5.60) in cellulose degradation than others, followed by horse's cecum (5.41) and then elephant's dung (5.30) (Table 4). Buffalo's colon fungi seem higher activity than fungi from horse digestive tract and elephant's dung, but there were not significant.

Table 1. Number of lignocellulolytic colonies in 10^{-3} dilution

No.	Isolate source	Code	Colonies			Total of colonies
			Lignin degrader	Xylan degrader	Cellulose degrader	
1.	Buffalo's rumen	Rkb	1**	5+14*	27+236*	283
2.	Buffalo's cecum	Skb	5**	0 +8*	10+222*	246
3.	Buffalo's colon	Kkb	3**	0+19*	15+326*	363
4.	Horse's cecum	Skd	1**	0+17*	12+242*	272
5.	Horse's colon	Kkd	4**	1+13*	6+124*	148
6.	Elephant's dung	Fg	2**	1+13*	4+142*	162

*clear zone around colony, **diffusion zone around colony. Rkb=Buffalo's rumen, Skb=Buffalo's cecum, Kkb=Buffalo's colon, Skd=Horse's cecum, Kkd=Horse's colon, Fg=Elephant's dung

Table 2. Lignolytic activities

Isolate source	Colony's color	Colony diameter (mm)	Diffusion zone diameter (mm)	Activity ratio
Buffalo's rumen	Brown	7.63	17.63	2.31
Buffalo's cecum	Brown	7.80	16.67	2.14
Buffalo's colon	Brown	7.52	17.70	2.35
Horse's cecum	Brown	7.40	17.63	2.38
Horse's colon	Brown	7.38	16.30	2.21
Elephant's dung	Green grey	5.92	13.60	2.29

Even though it's was not significant these data strengthen the Puppo et al. (2002) and Wanapat (2001) statement that buffalo has higher fiber degrading fungi than others animal farming. From buffalo point of view itself, cellulolytic from colon is superior than fungi from rumen (4.77) or cecum (4.38). This study also prove the hypothesis that lignocellulolytic from colon has higher activity in fiber degradation. Profile of lignin, xylan and cellulose degrading fungi base on morphological analysis showed in Figure 1.

Quantitative Analysis

Several lignin, xylan, and cellulose degrading fungi were superior than others (Table 5). It was concluded from quantitative analysis of enzyme activities that the highest lignolytic isolates was isolated from horse's cecum, xylanolytic was from horse's cecum, and cellulolytic was from buffalo's colon. The enzyme activities of ligninase, xylanase and cellulose were 0.166 U/g protein, 5.037 U/g protein, and 2.488 U/g protein respectively. The data showed positive correlation with qualitative analysis based on each activity ratio. Qualitatively, lignolytic isolates of horse's cecum, xylanolytic isolate of horse's cecum and cellulolytic isolate of buffalo's colon were the best selected among isolates, with activity ratio 2.38, 6.67 and 5.60 respectively (Table 2, 3, 4).

Quantitative and qualitative test for ligninase, xylanase and cellulase enzymes activities showed that selected lignocellulolytic fungi was found more frequently from cecum and colon both from buffalo and horse. It's strengthen the hypothesis above that lignocellulose degrading fungi 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 has similar composition microbes with ruminants. Futhermore this study showed that the cecum and colon lignocellulolytic fungi have tended higher ability to degrade lignocellulose than rumen fungi.

The three selected fungi then were tested for classification up to genus. Figure 1 showed the colony shape of lignolytic isolates of horse's cecum, xylanolytic isolate of horse's cecum and cellulolytic isolate of buffalo's colon. The three isolates have similarities. Color of colony are grey-green to brown-green, while the spores are black. Conidiophore arising from a foot-cell in the mycelium. The fruiting hyphae have enlarge to form a rounded globular tip. From the enlarge tip arise stigmata which produce a chain of conidia. According to the microscopic and macroscopic analysis by fungi classification, the three of isolates were tend to be *Aspergillus sp* genus (Machida and Gomi, 2010). Milala et al. (2009) showed that *Aspergillus sp* genus have a potentiality of converting lignocellulose material into product of commercial and industries value. Species of Aspergilli are known to produce all three enzyme activity of cellulases complex and exhibit strong hydrolytic activity (Mathew et al., 2008). *Aspergillus niger* genus could apply to degrade some local lignocellulose to produce reducing sugar (Ja'afaru and Fagade, 2007). Emtiazi et al., (2001) also showed that biodegradation of lignocellulosic waste improve by *Aspergillus terreus.*

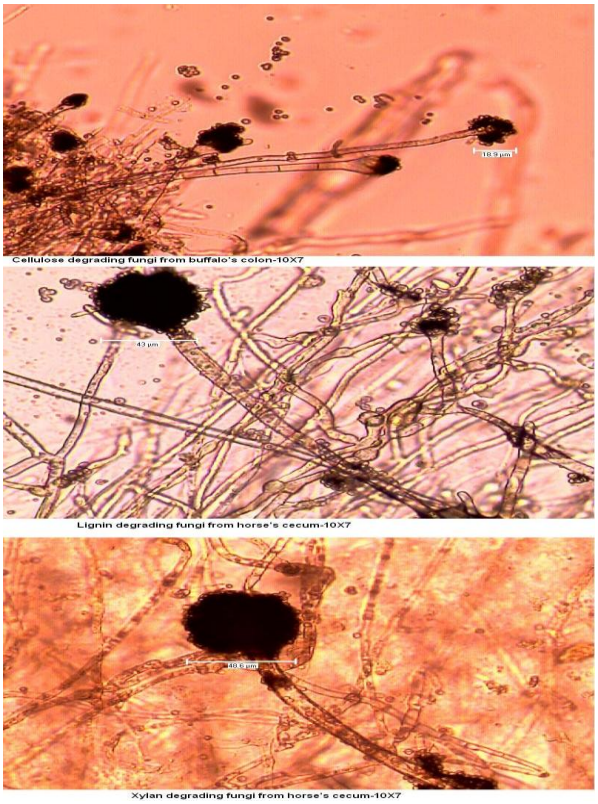

Figure 1. Morphology of selected lignocellulolytic fungi by Optilab camera with 280x**.** A = Lignin degrading fungi from horse's cecum, B = Xylan degrading fungi from horse's cecum, C = Cellulose degrading fungi from buffalo's colon

Table 3. Xylanolytic activities

Isolate source	Colony' color	Colony diameter (mm)	Clear zone diameter (mm)	Activity ratio
Buffalo's rumen	Green brown	2.73	15.87	5.81
Buffalo's cecum	Green brown	2.67	16.15	6.05
Buffalo's colon	Green brown	2.72	15.73	5.78
Horse's cecum	Green brown	2.18	14.55	6.67
Horse's colon	Green brown	2.65	15.10	5.70
Elephant's dung	Green brown	2.68	15.80	5.89

Table 4. Cellulolytic activities

Isolate source	Colony' color	Colony diameter (mm)	Clear zone diameter (mm)	Activity ratio
Buffalo's rumen	Green brown	3.00	14.30	4.77
Buffalo's cecum	Green brown	3.55	15.53	4.38
Buffalo's colon	Green brown	2.53	14.17	5.60
Horse's cecum	Green brown	2.88	15.58	5.41
Horse's colon	Green brown	2.95	13.10	4.44
Elephant's dung	Green brown	2.90	15.37	5.30

Table 5. Enzyme activities (unit/g protein)

Isolate	Ligninase	Xylanase	Cellulase
Buffalo's rumen	0.021	3.488	1.073
Buffalo's cecum	0.049	2.302	1.290
Buffalo's colon	0.162	0.798	2.488
Horse's cecum	0.166	5.037	2.437
Horse's colon	0.085	3.382	2.278
Elephant's dung	0.064	1.812	0.915

Table 6. Dry matter (DM), organic matter (OM), crude fiber (CF), non-digestible fiber (NDF) and acid digestible fiber (ADF) digestibility (%)

No	Isolate	Digestibility				
		DM	OM	CF	NDF	ADF
1.	Ruminal fluid	29.68[a]	33.92[a]	26.44[a]	25.50[a]	39.35[a]
2.	Ruminal fluid + *Aspergillus sp* (A)	31.03[b]	35.43[b]	28.21[b]	28.47[b]	42.59[b]
3.	Ruminal fluid + A+*E. casseliflavus*	30.76[b]	35.49[b]	29.09[b]	26.03[a]	39.92[b]
4.	Ruminal fluid + A+*B. licheniformis*	30.70[b]	34.42[a]	27.14[a]	27.56[b]	39.57[a]
5.	Ruminal fluid + A+*W. paucula*	31.30[b]	34.83[b]	26.08[a]	25.57[a]	42.86[b]

Inoculum's Administration for Rice Straw Degradation

Administration of *Aspergilillus sp* as inoculum for rice straw degradation could increase dry matter (DM), organic matter (OM), crude fiber (CF), netral detergent fiber (NDF), and acid detergent fiber (ADF) degradability (Table 6).

Adding *Aspergilillus sp* inoculum into rumen fluid medium elevated significantly DM, OM, CF, NDF, and ADF digestibility as 4.55%, 4.45%, 6.69%, 11,65% and 8.23% respectively. Paul et al. (2004) showed that administration of anaerobic gut fungus isolated from wild ruminant's feces increased the total of dry matter digestibility (DM, OM, NDF and ADF). Pugalenthi and Parimelazhagan (2010) also showed that utilization of rumen fungal isolate could increase the NDF digestibility of lignocellulose feed after 48 hour incubation. Cellulase enzymatic activity of *Aspergilillus niger* isolated from waste paper material were higher than those of fungal strain of the genus Trichoderma, known as high cellulase-

producing fungi (Peciulyte, 2007). Using *Aspergilillus sp* inoculum co-culture with lignocelulose degrading bacteria (*Enterococcus casseliflavus, Bacillus licheniformis* and *Wautersia paucula*) in general also increase all fiber fraction digestibility although as not good as *Aspergilillus sp*. Dashtban et al. (2009) showed that fungal co-culturing offers a means to improve hydrolysis of lignocellulosic residues. These data showed that administration of lignocellulose-degrading microbes was important to improve fiber digestion in rumen. Krause et al. (2003) also showed that fiber degestion in rumen was influenced by amount of fiber-degrading microbes.

Conclusions

Lignocellulose degrading fungi could be found from all samples in this study. Based on quantitative and qualitative selection, lignolytic isolates from horse's cecum, xylanolytic isolate from horse's cecum and cellulolytic isolate from buffalo's colon were the selected isolates. The lignolytic isolates of horse's cecum, xylanolytic isolate of horse's cecum and cellulolytic isolate of buffalo's colon were predicted as *Aspergillus sp* genus. Administration of *Aspergilillus sp* inoculum into rumen fluid medium elevated significantly DM, OM, CF, NDF, and ADF digestibility as 4.55%, 4.45%, 6.69%, 11.65% and 8.23% respectively.

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