African Journal of Biotechnology Vol. 9(20), pp. 2994-2999, 17 May, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Potency of fibrolytic bacteria isolated from Indonesian sheep's colon as inoculum for biogas and methane production

A. Wahyudi[1]*, L. Hendraningsih[2] and A. Malik[2]

[1]Center for Biotechnology Development, Muhammadiyah University of Malang, Indonesia.
[2]Department of Animal Production, Muhammadiyah University of Malang, Indonesia.

Accepted 2 April, 2010

Three fibrolytic bacteria were isolated from sheep's colon using cellulose (b), xylan (c) and lignin (d) as selective substrates. The potency of fibrolytic was identified by Subbarao methods. These isolates were then used both in pure and mixed culture with cattle cellulolytic bacteria (a) from the previous research. The isolates were subsequently selected based on their ability to produce gas through biogas and methan measurement using *in vitro* gas test technique of Tilley and Terry methods. Based on the parameter, the potential isolates then were applied as inoculum for dairy cattle feces fermentation. The application research was based on Randomized Completely Design with inoculum concentration (0, 3, 6 and 9%) as the treatment with three replications. The result showed that the highest *in vitro* biogas and methane production was obtained from a-c-d co-culture addition. The a-c-d co-culture as inoculum for *in vitro* feces fermentation could increase gas production 56.36% and methane 18.09% compared to natural fermentation by feces microbes. Application of 3 to 9% of a-c-d inoculum at the fermentation scale was highly significant (P < 0.01) in increasing biogas production, however, addition of more than 3% of a-c-d inoculum decrease methane concentration. The fibrolytic bacteria from sheep's colon were potential to increase the efficiency of biogas and methane production; thus, it should be considered as an inoculum for anaerobic fiber waste fermentation.

Key words: Fibrolytic bacteria, sheep's colon, inoculum, biogas, methane.

INTRODUCTION

Animal organic fibre waste could be processed into biogas as an alternative energy to replace fossil oil. The main poblems of biogas production were ineffective fermentation and low concentration of methane. The natural fermentation of inhabitant microbial feces was seen as the primary reason. Inoculum to increase fermentation effectivity was very important and could be used to increase biogas and methane production, yet it was unavailable in Indonesia. Rumen microbes ecology show that intensive farming system made ruminants unable to get enough supply of fibrolytic bacteria from soil and grass' roots that had important role in lignocellulose biodegradation as thebiogas precursor. Thus, the biogas produced from fermentation of animal feces in biodigester was lower than the expectation. In Indonesia, sheep is ruminant and is generally raised by grazing. Therefore, it had high opportunity to get a fibrolytic bacteria, which was the inhabitant bacteria in sheep's gut and could be used as the source for biogas production.Cellulose in lignocellulose complex form is undegraded by cellulolytic bacteria. Lignocellulose had very strong covalent bond that could not be hydrolyzed by cellulase. This complex bond could only be degraded by extra celluler enzyme that secreted by lignolytic microbes bacteria that is, phenol oxidase, (laccase), and peroxidases lignin peroxidase (Lip) and Manganase peroxidase (MnP) (Howard et al, 2003). These enzymes would break methoxyl double bond from lignocellulose stucture and decrease the methoxyl and increase hydroxyl and carboxyl phenolate groups. Lack of lignolytic bacteria in digester makes uncomplete lignocellulose degradation. For fibre degradation effectiveness, another group of fibrolytic bacteria (lignolytic and xylanolytic) were needed

*Corresponding author. E-mail: wahyudi_biotek@yahoo.co.id. Tel: (0341) 464318. Fax: (0341)460782.

to be added into the digester. The fibre degradation, especially lignin fraction, had to be increased to produce cellulose and lignin derivate as precursor of biogas and methane. The optimation of lignolytic, cellulolytic and xylanolytic bacteria formulation from sheep's colon was conducted to get an optimum fermentation inoculum.

MATERIALS AND METHODS

Sample collection

The main material in this study was fresh colon sample of local Indonesian sheep from the slaughtering house. Rumen fluid and isolate of cellulolytic bacteria from cattle was used as the comparison. Colon fluid sample was obtained from gut right after sheep's death by filtering into a prewarm (39°C) thermos flask (Lowe, 1986). Oxygen was removed from gas by filling CO_2 into flask and covering it with a sterile butyl rubber stopper. Diluted sample in series of 10-fold dilution or applied as soon as possible after specimen collection.

Medium for selection

Microbes were cultured by anaerobic methods (Hungate, 1957 in Ogimoto and Imai, 1981) in a selective solid medium containing 0.02 g KH_2PO_4, 0.03 g K_2HPO_4, 0.01 g $MgSO_4$, 0.01 g $CaCl_2$, 0.10 g NaCl, 0.10 g $(NH_4)_2SO_4$, 0.10 ml rezasurin 0.1%, 0.02 g cystein-HCl.H_2O, 0.40 g Na_2CO_3, 30.00 ml rumen fluid, 1.00 g substrate (cellulose/xylan/lignin), 2.00 g agar and 70.00 ml aquadest. All mineral solutions, (except cystein-HCl.H_2O and Na_2CO_3), resazurine solution, agar and destilated water were placed in a 150 ml round bottomed flask and boiled to dissolve agar. Then the rumen fluid added as a media (Bachruddin, 1985) and oxygen-free CO_2 gas passed into the medium at 45 to 60°C in the water bath. The flask covered with a rubber stopper and wired with wire loop. The medium sterilized in the autoclave at 121°C for 20 min. After sterilization, the medium cooled to 45 to 50°C in a water bath and passed oxygen-free CO_2 gas again through it. Cystein-HCl.H_2O, substrate and Na_2CO_3 solution added later. The medium mixed in the flask and transferred aseptically to a sterile tube with oxygen-free CO_2 gas displacing all air. Then 10^{-7} diluting colon fluid inoculated into the tube and closed with a sterile butyl rubber stopper. The tube spanned to produce a thin layer of agar on the tube wall. The culture then incubated in 39°C during 7 - 14 days.

Isolation of colonies

From these inoculated roll tube containing the selection medium, the individual colonies of fibrolytic bacteria pricked. A minimum of 10 isolates from each sample carefully pricked using a bent platinum-iridium needle. The bacteria then transferred to plate agar medium anaerobically with gassed oxygen-free CO_2 in an anaerobic jar. The plates incubated at 39°C during 5 - 7 days. As soon as sufficient growth reached, the colonies identified for its color, diameter of clear and diffusion zone (Subbarao, 1993; Martani et al., 2003) to choose the potential isolates.

Medium for gas test

The McDougall buffer solution and *in vitro* gas test culture were made by Tilley and Terry methods (Dayananda et al., 2007). Each litre consist of 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. All materials were soluted in Erlenmeyer flask and warmed until 39°C and passed by CO_2 gas, the pH was at 6.8. Sample of 0.5 g rice straw meal (2 mm in size) + 7 ml dairy cattle feces and 28 ml McDougall solution (1: 4) were placed into syringe bottle (volume 50 ml), shaked and CO_2 passed through it. As treatment, 10% of each inoculum was added to substitute the rumen liquid. The bottle then was closed strictly and incubated at 39°C for 72 h. Gas production was collected in venoject 1, 12, 24, 48 and 72 h incubation. Methane concentration meassured was by gas chromatography (GC).

Dairy cattle feces fermentation

Six kilograms of fresh dairy cattle feces with six liter aquadest (1:1) were poured into a pail then homogenized through stirring. The mix solution was then divided into 12 sterile bottles. Each bottle through which CO_2 gas was passed for 10 min and the best mix inoculum (3, 6 and 9%) added. Each bottle was closed by a plastic in vacuum condition to hold the gas production. Each treatment was replicated three times. The bottles were then incubated in water bath at 39°C. The biogas production and methane concentration was studied for 5 weeks.

RESULTS AND DISCUSSION

Production of biogas

Biogas production in 72nd h incubation showed in Figure 1. According to Figure 1, the highest biogas production was reached by combination addition of a, c and d isolates co-culture. The biogas production of this co-culture indicated that the mixed inoculum (cellulolytic, xylanolytic and lignolytic) bacteria from sheep's colon gave optimal condition for natural faecal microorganisms. Biogas production in an anaerobic fermentation represented the activity of fiber digestion, thus, the addition of cellulolytic, xylanolytic and lignolytic bacteria in the medium would increase fecal-lignocellulose break-down and degraded it to more simple fraction.

The second and third highest biogas production were reached from combination of a-b-d and b-c-d, respectively. This phenomenon showed that the (lignolytic bacteria) was an important factor in crude fibre degradation and biogas production. Lignolytic bacteria opened the lignin structure that is protecting xylan and cellulose in lingo-cellulose complex. Xylan and cellulose could only be hydrolyzed if lignin structure is broken (Migne et al., 1996). Three isolates (lignolytic, xylanolytic and cellulolytic) availability in fermentation medium simultaneously made better crude fibre degradation and also biogas production. Akin and Benner (1988) reported that rumen bacteria had high activity to degrade lignin and change it into biogas. This study also showed that colon lignolytic was superior to produce biogas. Similar result exists with biogas production at 72nd h (Figure 2). The highest biogas production was reached with a-c-d co-culture addition (86 ml). This also showed that lignolytic had important role in increasing biogas production. Using a-c-d as inoculum, combination of dairy cattle feces fermentation,

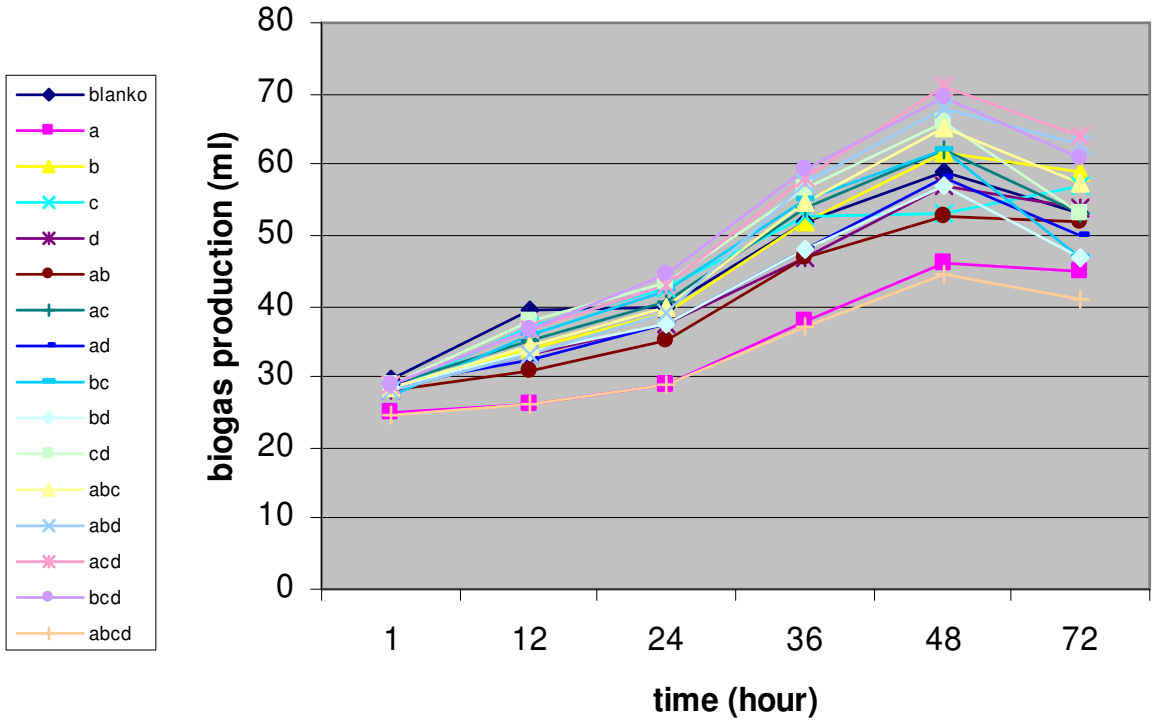

Figure 1. Biogas production profile. Blanko = faeces microbial, a = cattle rumen cellulolytic isolate, b = sheep colon cellulolytic bacteria, c = sheep colon xylanolytic bacteria and d = sheep colon lignolytic bacteria.

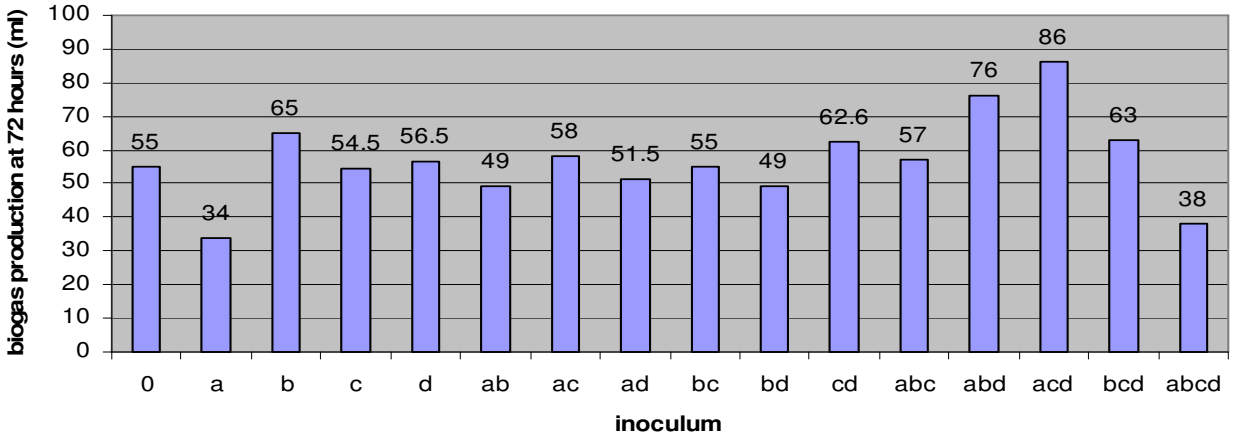

Figure 2. Gas production at 72nd h. Blanko = faeces microbial, a = cattle rumen cellulolytic isolate, b = sheep colon cellulolytic bacteria, c = sheep colon xylanolytic bacteria and d=sheep colon lignolytic bacteria.

in this study could increase 56.36% gas production compared to natural fermentation of inhabitant microbes' feces. According to Basuki (1992) and Ahring (2003) addition of fermentation inoculum in digester could increase the biogas production.

Production of methane

Figure 3 showed methane production in 72nd h of incubation. Similar pattern also existed in this parameter. The highest methane production was obtained through co-culture a-c-d addition (18.09%).

A study of lignocellulose anaerobic degradation by rumen bacteria showed that lignin was a precursor of methane (Colberg, 2001). Similar result was showed in this study that lignin was an important thing in feces degradation into methane. Lignin could only be degraded by oxidative system and depolimerization by secretion of lignase extra cellular (Peres et al., 2002). Lignocellulolytic

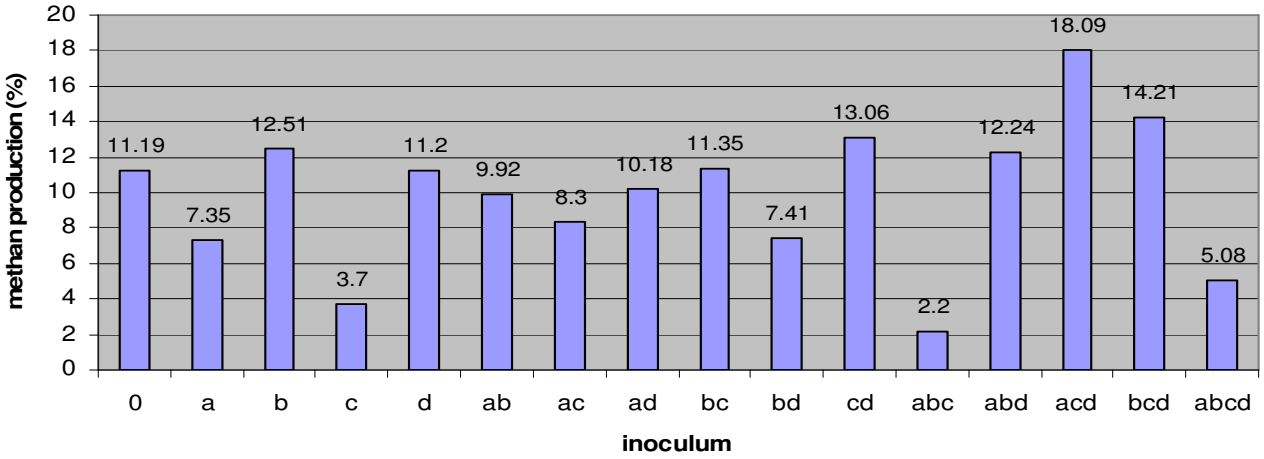

Figure 3. Methane production at 72nd h. Blanko = faeces microbial, a = cattle rumen cellulolytic isolate, b = sheep colon cellulolytic bacteria, c = sheep colon xylanolytic bacteria and d = sheep colon lignolytic bacteria.

degrader bacteria was considered exist in rumen, cecum, or colon (DeGregorgio et al., 1984; Ulrey et al., 1997).

Increasing feces crude fibre degradation by a-c-d co-culture addition would supply precursor for methane forming. The final product of complete anaerobical crude fibre degradation was volatile fatty acid (VFA), CO_2 and also H_2 as precursor of methane. Using a-c-d co culture as inoculum for dairy cattle feces fermentation in this research could increase 61.66% methane production compared to the natural fermentation of inhabitant microbial feces.

Inoculum's application

Production of biogas

Application of a-c-d as inoculum in scaling up dairy cattle feces fermentation showed that the increase in production of biogas highly significant ($P < 0.01$). Biogas was produced more efficient with a-c-d inoculum addition than control. The efficiency was biogas volume elevation and shorter fermentation time. Using 3 to 9% of a-c-d inoculum as starter could increase biogas production one or two times; in addition, it was two weeks faster in reaching the peak of biogas production. Using a-c-d inoculums also shortened the lag phase from 3 weeks into 1 week (Figure 4). This result proved the previous idea that biogas production could be harvested earlier and increased by inoculums addition.

Inoculum addition supplied active starter that could reduce lag phase duration. Long lag phase periode must be avoided since it consumed time of production, made nutrient unused immediately by microbes for growth and give opportunity to be contaminated by other unexpected microbes. Lag phase was influenced by fermentation inoculums concentration and its physiological condition. Usually, 3-10% inoculums was used in fermentation; bacteria as fermentation inoculums must be used at growth logarithmic phase when cell is metabolically active. Adding fermentation inoculum at the end of logarithmic phase would create long lag phase (Stanbury et al., 2003).

In cellulase production process, lag phase of fermentation could be limited by using starter medium that has similar composition with growth media and contain active growth inoculum. Active inoculum was needed in enzyme production. To avoid the failness, 25% from end fermentation media could be used for latter fermentation. The advantage of active inoculum was higher than the disadvatage of contamination and microbes ability decreased. The fermentation inoculum advantage was also needed in biogas forming process (Basuki, 1991; Soejono et al., 1990).

Methane production

Methane concentration from treatments that measured in the fifth week showed highly significant ($P < 0.01$) result. Methane production from 0, 3, 6 and 9% inoculums addition were 13.55, 13.69, 9.90 and 0.0005%, respectively (Figure 5). Increase of the inoculum concentration decreased methane production. In contrast, biogas production increased with inoculum concentration addition (Figure 4). The data showed that optimal methane concentration was reached in 3% inoculum addition. The methane decreasing along the fibrolytic inoculum addition was caused by mechanism of feedback inhibition. Organic matter that degraded too fast would produce more soluble carbohydrate. The process makes accumulation of CO_2 and H_2 in medium and followed by depressing pH to toxic for fibrolytic bacteria. Production of biogas by 9% inoculum decrease dramaticaly in the

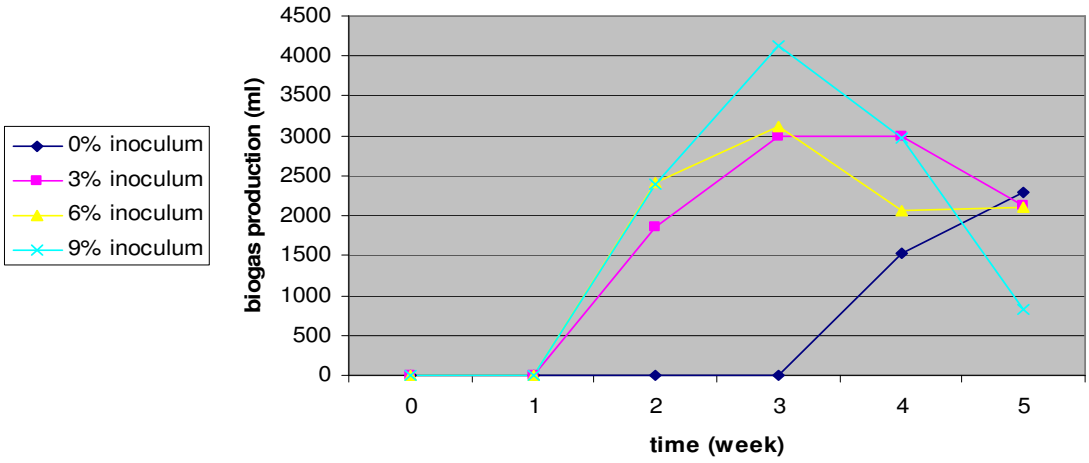

Figure 4. Biogas production using a-c-d inoculum.

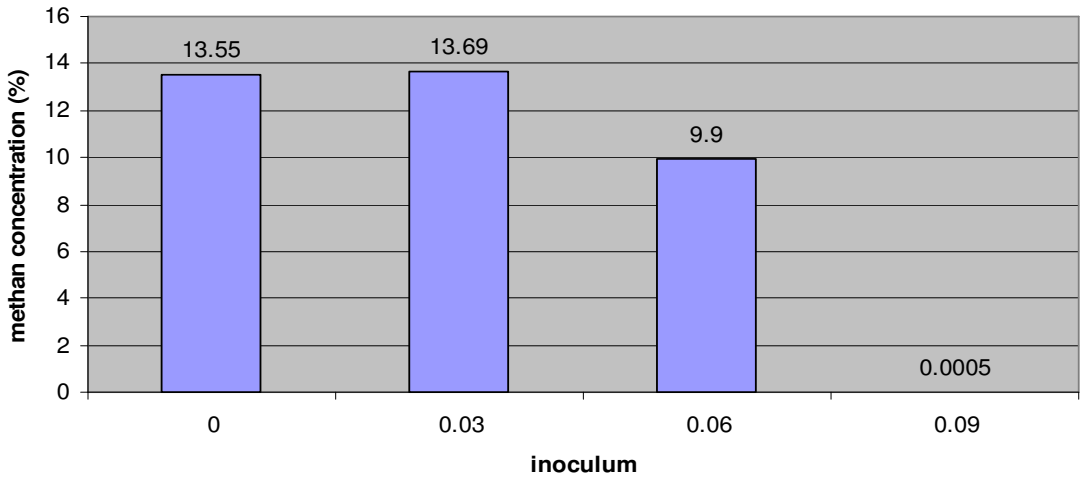

Figure 5. Methane concentration using a-c-d inoculum

fifth week. At the beginning, organic acid former bacteria were very actively degraded carbohydrate, fat and protein. These bacteria grew and multiplied very fast. Organic acid that are important for biogas forming were volatile organic acid, especially acetic acid, CO_2 and H_2. When organic acid forming is not followed by activity of bacteria that use acetic acid, CO_2 and H_2, the fibrolytic bacteria would be inactive.

Ahring (2003) stated that fast volatile fatty acid production must be balanced by addition of microbes that use acetic acid and hydrogen so that crude fibre degradation continued and pH did not decrease. Fibrolytic bacteria could only live in narrow pH range, 6 - 7 (Weimer et al., 1999).

Conclusion

In general, sheep's colon fibrolytic bacteria could increase the efficiency of biogas and methane production. Addition of a-c-d inoculum resulted in the highest biogas and methan production. Three percent of a-c-d inoculum was the optimum concentration for biogas
and methane production in anaerobic dairy cattle feces fermentation.

ACNOWLEDGEMENT

This publication is part of Competitive Grant Research funded by Directorate of Higher Education, Ministry of National Education Republic of Indonesia.

REFERENCES

Ahring BK (2003). Perspectives for anaerobic digestion. Adv. Biochem. Eng. Biotechnol. 81: 1-30.
Akin DE, Benner R (1988). Degradation of polysaccharides and lignin

by ruminal bacteria and Fungi. Appl. and Environ. Microbiol. pp. 1117-3655.
Bachruddin Z (1985). Development of Ruminal Microflora in Goat (*Capra hircus),* Thesis Program Pasca *Sarjana*, University of Philipines, Los Banos.
Basuki P (1991). *Aplikasi Biokonversi Limbah Organik untuk Produksi Gas Methan dan Peningkatan Kualitas Lingkungan Hidup*. PAU-Bioteknologi UGM. Yogyakarta.
Basuki P (1992). *Aplikasi biokonversi limbah organik untuk produksi gas bio dan peningkatan kualitas lingkungan hidup, Kursus singkat penanganan limbah industri*, PAU-Bioteknologi UGM 25 November 1991-15 Februari 1992.
Colberg PJ (2001). Microbial Degradation of Lignin-derivat Compound Under Anaerobic Conditions, Stanford University Publ. USA.
Dayananda TL, Nagpal R, Puniya AK, Sehgal JP, Singh K (2007). Biodegradation of urea-NH_3 treated wheat straw using anaerobic rumen fungi. J. Anim. Feed Sci. 16: 484-489
DeGregorio RM, Tucker RE, Mitchell GE, Gill WW (1984). Acetate and propionate production in the cecum and proximal colon of lamb, J. Anim. Sci. 58(1): 203-207
Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S (2003). Lignocellulose Biotechnology : issues of bioconversion and enzyme production. Afr. J. Biotechnol. 2 (12): 602-619.
Lowe SE (1986). The Physiology and Cytology of Anaerobic Rumen Fungus, A Thesis Submitted to the University of Manchester for the Degree of PhD. Department of Botany, Faculty of Science.
Martani E, Haedar N, Margino S (2003). *Isolasi dan karakterisasi bakteri pendegradasi lignin dari beberapa substrat alami*. Gama Sains, 2: 32-35.
Migne C, Grenet E, Jamot J (1996). Microbial degradation of the apical internode of Co125 and W401 maize in the rumen. Anim. Feed Sci. Technol. 58: 165-185.
Ogimoto K, Imai S (1981). Atlas of Rumen Microbiology. Japan Scientific Societies Press, Tokyo.
Peres J, Munoz-Dorado J, de la Rubia T, Martinez J (2002). Biodegradation and biological treatment of cellulose, hemicellulose and lignin: an overview. Int. Microbiol. 5: 53-56.
Soejono M, Sutariningsih E, Basuki P, Utomo R, Harsoyo (1990). *Pengaruh Amoniasi Ures Jerami Padi terhadap Kotoran Sapi untuk Produksi gas Methan*. PAU-Bioteknologi UGM, Yogyakarta.
Stanbury PF, Whitaker A, Hall SJ (2003). Principles of Fermentation Technology. Butterworth-Heinemann, Oxford.
Subbarao NS (1993). Biofertilizers in Agriculture and Forestry, 3th ed. International Science Publisher, New York.
Ulrey DE, Crissey SD, Hintz HF (1997). Elephants: Nutrition and Dietary Husbandry, Michigan State University. East Lansing, Michigan 48824
Weimer PJ, Waghorn GC, Merten DR (1999). Effect of diet on population of three species of ruminal cellulolytic bacteria in lactating dairy cows. J. Dairy Sci. 82: 122-134.