Indon. J. Trop. Agric. 7(1) : 5 - 9. 1996.

DNA RECOMBINANT TECHNIQUE FOR PRODUCING TRANSGENIC RUMEN MICROBES IN ORDER TO IMPROVE FIBER UTILIZATION

I. ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC BACTERIA[1]

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ABSTRACT

The cellulolytic bacteria isolated from the buffalo and cattle rumen - *Ruminococcus albus*, *R. flavefaciens*, and *Bacteroides succinogens* - had various activities. The capability of these bacteria to degrade cellulose ranged from 12.7 to 43.2 % per day. The cellulolytic activity of the bacteria from the buffalo rumen was higher than that from the cattle rumen in pure as well as in mixed culture. Degradation rate of the buffalo rumen bacteria was 43.2 % per day while of the cattle rumen bacteria was only was only 16.3 % per day. In pure culture, the degradation rate of *R. albus* from the buffalo rumen was 21 % per day, whilst that from the cattle rumen was 12.7 %. It could be concluded that the superior character of the buffalo rumen bacteria was due to individual genetic factors as well as the synergetic activities of the bacterial population in the buffalo rumen. The amylolytic bacteria isolated from the rumen were *Streptococcus bovis* and *Bacteroides ruminicola*.

Keywords: Recombinant, cellulolytic, *R. albus*, *R. flavefaciens*, *B. succinogens*, amylolytic, *S. bovis*, *B. ruminicola*

Ruminants (cows, buffaloes, sheep. goats, etc.) are the meat and milk producers with high nutritive quality for human beings. These animals are able to convert high fiber feed into high quality meat and milk products through the microbial activity in the rumen. Since there are not enough pasture in Indonesia, these animals greatly depend on the agricultural and agro-industrial waste products in the form of lignocellulose difficult to digest by the rumen microbes. This is one of the reasons for the low animal productivity in Indonesia.

The technology that could overcome this problem is through the development of biotechnology, i.e., creating transgenic microbes in the rumen with high cellulolytic capacity. The farmers do not need to process the ligno-cellulose wastes, since the animals have high capability to digest wastes through genetic engineering. Hesspel (1985) reported that the high cellulolytic capacity of the rumen microbes could be improved through genetic engineering. Little improvement of the fiber digestibility by cellulolytic microbes could significantly increase the animal growth. Therefore, feed biotechnology would be better stressed on generating the microbes with high cellulolytic activity.

Buffaloes are farm animals rarely used in research programs in Indonesia, although they have many superior characteristics: they are very appropriate for Indonesia tropical wet climate, and they have high activity of cellulolytic microbes in the rumen. Sutardi (1978) proved that cellulolytic digestibility in the rumen of the buffalo was higher than that of the cow.

Through this research, transgenic microbes with high cellulolytic activity would be created and be responsive to concentrate feeding improvement. The sources of the cellulolytic genes were obtained from buffaloes, transformed to the non-cellulolytic microbes in cattle rumen that have high growth rate and population.

This research has been planned for four years. During the first year are to be conducted the isolation, selection, and identification of cellulolytic bacteria from the buffalo and cattle rumens. In the second year, the activities will include identification and characterization of the genetics of the target bacteria. Whereas during the third year, the interest is to transfer the DNA responsible for generating transgenic bacteria. The fourth year will comprise the evaluation of nutritive performances, survival rate, and competitive transgenic bacteria as well as their applications in the field. This report contains the results of the experiments carried out in the first year of the four year research plan.

MATERIALS AND METHODS

Experiment-1: Isolation and Identification of Cellulolytic Bacteria

The isolation of cellulolytic bacteria was conducted according to Bauchop and Mountfort (1981), and Bauchop (1989). The rumen fluid from the stomach tube, or directly from the fistula, was filtered with a two layer gauze and placed in a vacuum flask in order to maintain the right

[1] Research funded by the Indonesia National Research Council (DRN) and presented in the Second International Conference on Agricultural Biotechnology, Jakarta - Indonesia, June 1995

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temperature. To give the opportunity for the cellulolytic bacteria to grow, but depress others, the inoculum was first cultured in a cellulose medium as its energy source. Five percent of the inoculum was transferred into 5 ml of standard liquid medium containing cellulose, the only energy source or carbon source. It was then incubated at 39 ^{0}C for the bacteria to grow. The decreasing amount of cellulose in the medium indicated the growth of the bacteria. From the most active culture, 0.05 ml was transferred into the solid medium consisting of agar and cellubiose mixture and packed using the "role tube technique" (Hungate, 1966). The packed medium was then reincubated at 39 ^{0}C. After culturing for 3 to 5 d, the colony was transferred into the cellulose liquid medium. In order to obtain a pure isolate, the culture from the cellulose liquid medium was retransferred into the cellobiose solid medium. The pure culture was identified by its uniformity of the colony growth in the medium, morphological uniformity of the bacteria, and size as well as color (by Gram staining of the bacteria and determined microscopically).

The bacteria were cultured anaerobically at 39 ^{0}C in a medium (according to Hungate, 1966) using pure carbon dioxide. The mineral solution consisted of solution A (KH_2PO_4, 0.3 %; NaCl, 0.6 %; $(NH_4)_2SO_4$, 0.3 %; $CaCl_2$, 0.03 %; and $MgSO_4$, 0.03 %) and solution B (K_2HPO_4, 0.03 %). The composition of the medium consisted of 165 ml solution A, 165 ml solution B, 170 ml free cell rumen liquid (sterile), 500 ml distilled water, 5 g $NaHCO_3$, 1 g yeast extract, 1 g peptone (Difco), 0.2 g cysteine-HCl, and 0.001 g resazurin (anaerobic indicator). The medium was transferred anaerobically into the culture tubes (160 X 13 nm). The tubes were closed with rubber cap, and sterilized in an autoclave. The pH of the medium ended at 6.9.

The culture technique to maintain and subculture the bacteria was done according to Hungate (1966). One twentieth of the medium volume cultured for 4 to 5 d was inoculated in a new medium with the same composition.

Beside the morphological observation of the isolate, the biochemical analysis particularly on the metabolism product of each isolate obtained from different substrates was also conducted. The isolates were identified according to Holdeman and Moore (1972), and Ogimoto and Imai (1972). The volatile fatty acids (VFA) in the media were determined by means of filtration. One milliliter of the precipitant solution, consisting of methaphosphoric acid and distilled water, was added into 4 ml of the substrate. It was then centrifuged at 3500 rpm for 30 minutes and analyzed using gas chromatography method by injecting 2 μl of the supernatant. The condition of the column was as followed : column temperature = 120 ^{0}C; detector temperature = 200 ^{0}C; injector temperature = 180 ^{0}C; flow rate = 20 ml/minute; range = 11; and attenuation = 4. The formic and sucinic acids were determined qualitatively.

Experiment-2 : Isolation and Identification of Amylolytic Bacteria

A 100 ml medium was used to isolate the amylolytic bacteria. The composition of the medium consisted of 0.1 g peptone, 0.25 g yeast extract, 15 ml mineral solution A, 15 ml mineral solution B, 20 ml sterile rumen liquid, 2 g agar (Difco), 0.0001 resazurin, 0.5 g soluble starch, 0.05 g cysteine-HCl, 0.4 g $NaHCO_3$, and 50 ml distilled water. In order to detect the amylolytic activity, the culture medium (after transferring the colony into the different medium) was dipped into an iodine solution (KI solution).

The growth of the amylolytic bacterial isolate was measured *in vitro*. Into the standard medium (the same medium used for isolating the amylolytic bacteria which did not contain agar), where soluble starch served as the source of energy/carbon skeleton, the tested isolate was inoculated to observe its growth characteristic. Six Hungate tubes containing starch media (amylum) were inoculated with 0.1 ml of the tested cultured bacteria and then incubated at 39 ^{0}C. Six different time periods of incubation of 0, 15, 30, 45, 60, and 75 minutes were used. At the end of the incubation period, the bacteria were killed by adding one drop of saturated $HgCl_2$. The absorbent (optical density) was measured from each culture using double beam spectrophotometer at 600 nm wavelength. The more turbid the medium, the higher the population of the microbes.

Regression analysis to determine the growth rate and doubling time was done for each tested microbe. Other criterion used to evaluate the amylolytic activity was the acid production that could be measured from its pH (Hungate, 1966).

Experiment-3: Comparison Test on Cellulolytic Activitiy of Bacteria from Cattle and Buffalo Rumens

In the standard media consisting of 100 mg insoluble cellulose, the cellulolytic microbes tested for their activities were inoculated. They were then incubated at 39 ^{0}C in different time periods. The tested microbes were a mixed culture and a pure culture. The cellulose degradation was measured by gravimetric technique (Halliwell and Bryant, 1963). The cellulose degradation was followed by the weight determination of the undegraded cellulose using sintered filter glass, which was then rinsed with 5 ml HCl 4 N, NH_4OH 0.7 N. Ethanol was used as cleaning solution. Twenty milliliters of distilled water was always used to rinse the undegraded cellulose after rinsing with the cleaning solution. The clean undegraded cellulose was then dried at 60 ^{0}C for 24 h.

The cellulose medium without the test microbes inoculated was used as control. The cellulose degradation was expressed as the percentage of the degraded cellulose over the total amount of the cellulose in the medium.

In this experiment, the tested inocula were: (1) mixed culture from the cattle rumen microbes and (2) mixed culture from the buffalo rumen microbes. After isolation, purification, and identification, the isolate obtained was then determined for its cellulolytic activities.

RESULTS AND DISCUSSION

Experiment-1: Isolation and Identification of Cellulolytic Bacteria

The results of isolation and morphological as well as biochemical identification of the cellulolytic bacteria in buffalo and cattle rumens are shown in Table 1. The isolates listed are those having the cellulolytic activities where they could grow in the cellubiose agar media and could degrade cellulose in the liquid media. There were nine isolates, i.e., four isolates were derived from buffalo rumen and five isolates were derived from cattle rumen.

The isolates were obtained from 10^{-7} to 10^{-10} dilution. Hungate (1966) reported that isolation of the cellulolytic bacteria could be done from 10^{-6} to 10^{-7} dilution. The population of the cellulolytic bacteria was greatly depended upon the feed consumed by the hosts (ruminants). Giving high forage as usually consumed by buffaloes in Indonesia increased the bacteria population. Giving concentrate in the diet would decrease the cellulolytic bacteria population, but increased the amylolytic bacteria.

The populations of the cellulolytic bacteria in a cattle and a buffalo were 4.68 x 10^9/ml and 7.27 x 10^{10}/ml, respectively. The bacteria identified were : *Bacteroides succinogenes* (two isolates), *Ruminococcus flavefaciens* (four isolates), and *R. albus* (three isolates). Within the same species, different colors and capabilities in forming a "clear zone" in the cellubiose media were observed. In general, *R. flavefaciens* colony gave yellow color with different intensities depending upon the strains and the availability of the nutrient (Stewart and Bryant, 1988). The activities of these bacteria were very much dependent upon the pH of the rumen.

The capability in forming the "clear zone" was also influenced by the strains of the cellulolytic bacteria. Leatherwood (1973) reported that after culturing for 5 d in the media, some of the cellulolytic bacteria colony could form a "clearing zone" and some could not.

There were two kinds of bacteria found from this

Table 1. Isolation and Some Characteristics of Isolates of the Rumen Cellulolytic Bacteria from Buffalo and Cattle.

No	Origin	Colonies: Color	Colonies: Shape	Clearing Zone	Gram stain	Shape of Cells	Substrate/Energy source
	Buffalo						
1	10-8	white	rounded	+	+	cocci	cellulose
2	10-9	yellow	rounded	+	+	cocci	cellulose
3	10-10	white	rounded	-	+	cocci	cellulose
4	10-9	white	irregular	+	-	rod	cellulose
	Cattle						
5	10-7	white	rounded	+	+	cocci	cellulose
6	10-7	yellow	rounded	+	+	cocci	cellulose
7	10-10	white	irregular	+	-	rod	cellulose
8	10-7	yellow	rounded	-	+	cocci	cellulose
9	10-8	yellow	rounded	-	+	cocci	cellulose

Table 1. Continued

No	Fermentation end Product: A	P	iB	B	iV	V	F	Suc	Microorganism
1	+	-	-	-	-	-	+	-	*R. albus*
2	+	-	-	-	-	-	-	+	*R. flavefaciens*
3	+	-	-	-	-	-	+	-	*R. albus*
4	+	-	-	+	-	-	-	+	*B. Succinogens*
5	+	+	-	-	-	-	+	-	*R. albus*
6	+	-	-	-	-	-	-	+	*R. flavefaciens*
7	+	+	-	+	-	-	-	+	*B. Succinogens*
8	+	-	-	-	-	-	-	+	*R. flavefaciens*
9	+	-	-	-	-	-	-	+	*R. flavefaciens*

Abbreviations: A = Acetate; P = Propionate; iB = IsoButyrate; B = Butyrate; iV = Iso Valerate; V = Valerate; F = Formate; Suc = Succinate; Fermentation end Product = (+) or (-) reaction; R. albus = Ruminococcus albus; R. flavefaciens = Ruminococcus falvefaciens; B. Succinogens = Bacteroids succinogens.

experiment: (1) Gram-negative bacterium, i.e., *Bacteroides succinogenes* with rod shape and (2) Gram-positive bacteria, i.e., *R. albus* and *R. flavefaciens* with coccus shapes. All these bacteria were capable to degrade cellulose in the liquid media. The *B. succinogenes* formed a colony with irregular shape. It was suspected that this bacteria could migrate in the agar media, as so reported by Hungate (1966). The biochemical analysis showed that *R. flavefaciens* produced succinate while *R. albus* did not.

Experiment-2: Isolation and Identification of Amylolytic bacteria

The results of the isolation and identification of amylolytic bacteria in buffalo and cattle rumens are shown in Table 2. Two species of bacteria found in buffalo and cattle rumens could be identified, i.e., *Streptococcus bovis* and *Bacteroides ruminicola*, obtained from 10^{-9} to 10^{-10} dilution. *S. bovis* was the amylolytic bacterium that could increase in population as concentrate was given in the diet (Stewart and Bryant, 1988). The color of the colony varied from white, yellow, to orange. However, in the stock culture the color was always white (Hungate, 1966).

From this experiment, it was found that this isolate was able to grow in a starch medium as wel as in a glucose as the source of energy with lactate as the main product. Giving high concentrate in the diet caused acidosis due to some lactate accumulation by *S. bovis*. This bacterium has been identified as : Gram-positive cocci, white colony, capable of using starch and glucose as the main energy source and produce lactose and formic acid.

B. ruminicola was the amylolytic bacterium that could be identified. This bacterium digest starch, glucose, and xylose. *B. ruminicola* was one of the most numerous bacteria and was found in ruminants fed on many different diets (Steward and Bryant, 1988).

The growth analysis of the amylolytic bacterium is shown in Table 3. The growth rate of *S. bovis* from the rumen of the cattle was lower than of the buffalo. The comparison test of their growth rates on the liquid medium with starch as the main energy source is shown in Table 3. There was no great difference in the growth rate as well as

Table 2 Isolation and Some Characteristic of the Rumen Amylolytic Bacteria from buffalo and Cattle

No	Dilution	Colonies		Clearing Zone	Shape	Carbon - source						
		color	Shape			S	G	Gly	Xy	Su	Cb	Cl
	Buffalo											
1	10^{-8}	Orange	irregular	+	cocci	+	+	-	-	+	-	-
2	10^{-8}	white	rounded	-	rod to cocci	+	+	-	-	+	-	-
	Cattle											
3	10^{-7}	white	rounded	+	rod	+	+	-	+	+	-	-
4	10^{-7}	orange	irregular	+	cocci	+	+	-	-	+	-	-

Table 2 Continued

No	Fermentation end Product									Microorganism
	A	P	iB	B	iV	V	F	Suc	L	
1	+	-	-	-	-	-	-	-	+	S bovis
2	+	-	-	+	-	+	-	+	-	B. ruminicola
3	+	-	+	+	+	+	-	-	-	B. ruminicola
4	+	-	-	-	-	-	-	-	+	S bovis

Abbreviations : A=Acetate; P=Propionate; iB=IsoButyrate; B=Butyrate; iV=IsoValerate; V=Valerate; F=Formate; Suc=Succinate; L=Lactate; Fermentation end Product=(+) or (-) reaction. Carbon-source; S=Starch; G=Glucose; Gly=Glycerol; Xl=Xylose; Su=Sucrose; Cb=Cellubiose; Cl=Cellulose in the population doubling time of the microbes. It seemed that the animal factors (cattle vs. buffalo) did not influence the growth rate of the *S. bovis*.

Table 3 Growth Rate and Population Doubling Time of Streptococcus bovis of Rumen Buffalo Vs Cattle in Liquid Medium with Starch as Main Energy Source

Animal	Growth Rate (%/minute)	Population doubling time (minute)	Final pH
Cattle	2.86	26.3	5.37
Buffalo	2.99	23.1	5.37

Experiment-3: Comparison Test on Cellulolytic Activity of Bacteria from Cattle and Buffalo Rumens

The other objective of this experiment was to find the rumen bacteria with high cellulolytic activity. The cellulolytic activities were tested from the isolates obtained. The cellulolytic activity from the mixed culture of the bacteria in the rumen (cattle and buffalo) was also tested in order to find a synergetic activity among the bacteria in digesting cellulose (fiber). The result is shown in Table 4.

Table 4. Comparison Test of the Cellulolytic Activity of the Bacteria from the Cattle's and Buffalo's Rumen (n=4)

Bacterial Population	Animal	
	Buffalo	Cattle
	Cellulose degradation (%/day)	
Mixed Culture	43.2	16.3
Pure Culture (*R. albus*)	22.3	12.7

The cellulolytic activity of the bacteria in the buffalo rumen was higher than that of the cattle, whether it was obtained from a pure culture *(R. albus)* or from a mixed culture. In the mixed culture, the degradation in the buffalo rumen was 43.2 % per 24 h, while in the cattle rumen was 16.3 % per 24 h. Similar result was reported by Sutardi (1987) by using in vitro technique. In the pure culture (*R. albus*), the rate of the degradation was slower. The average rate of degradation in buffalo and in cattle were 21 % and 12.7 % per 24 h, respectively. *R. albus* was an important cellulolytic bacterium even though its activity was greatly dependent upon the strain (Bryant, 1973).

The difference in activity occurred in the mixed culture and in the pure culture indicated that there was a synergetic activity among the bacteria in the rumen (Leatherwood, 1973). Moreover, there were some differences in the activities of *R. albus* in buffalo as well as the differences due to the individual factors (genetic factors) of the bacteria. Within the same species, the different strains often determine the cellulolytic activity (Bryant, 1973). It could be concluded that the superior character of the cellulolytic bacterium from the buffalo rumen was due to the individual (genetic) factor of the bacterium as well as the synergetic activity in the population.

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