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Modification of the Pouch Method to Evaluate the Ability of Lactic Acid Bacteria Strains for Making Good Quality Silage in Thailand

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Abstract

To evaluate the ability of lactic acid bacteria (LAB) strains for making good quality silage in tropical regions, a silage fermentation model system was constructed by modifying a pouch method. *Enterobacter* sp. SG 1-1T and *Saccharomyces cerevisiae* SG 2-1Y, both isolated from Guinea grass silage, were used together with the LAB strain in the modified pouch method as typical silage coliform bacterium and yeast, respectively. For the silage material, autoclaved Napiergrass (*Pennisetum purpureum* Schumach.) was used. Two strains, LG 2-1 and N-22, used as model LAB strains grew well in this model system (modified pouch method) and their growth properties were strongly influenced by the inoculum size of each microorganism. From these results, this modified pouch method seems to be useful for the screening of LAB strains to make good quality silage in Thailand.

Discipline: Animal industry
Additional key words: yeast, coliform bacteria

Introduction

In Thailand, the amount of milk and fermented milk consumption has rapidly increased by about 2.5 times during the last 10 years mainly in urban areas due to an improvement in life style associated with the growth of the economy[5]. Furthermore, the promotion of milk for schoolchildren by the Thai government has been effective in increasing milk consumption. However, the self-sufficiency rate (SSR) in raw milk production (RMP) is low, accounting for about 60% of the demand. The reason for the low SSR seems to be due to immature feeding management based on the inexperienced history of dairy farming and the delayed introduction of techniques to dairy farmers in spite of the low milking ability of cows. The government (Department of Livestock Development) plans effective strategy for increasing RMP. The strategy includes improving cattle ability (breeding), feeds, cattle management and so on. The feeding of silage, a kind of stored forage fermented by lactic acid bacteria (LAB), is one of the effective and easily introduced techniques to increase RMP. It was reported that feeding of good quality silage throughout a year stably increased the average RMP in a project[8] by the Japan International Cooperation Agency. The average RMP in the project was about 1.5 times higher than that of Thailand[5,8]. Feeding of good quality silage seems to be a suitable way to increase RMP in spite of it not having wide use in Thailand. The fermentation quality of silage prepared by wild LAB is, however, very varying and sometimes very poor. The production of good quality silage is not always assured.

To cope with this problem, LAB strains suitable for making good quality silage in Thailand were screened and some thermophilic LAB strains were isolated from

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silage in Thailand[3]. These strains showed peculiar properties adapted to the tropical environment such as growth at high temperature (40–45ºC). The application of these strains to actual silage preparation has not yet begun because no evaluation system for silage-making with LAB in Thailand has been developed. Therefore, we tried to construct an evaluation system for LAB strains. A model fermentation system for making good quality silage in Thailand was modified from a pouch method. The original one[4,6] was developed in Japan for screening of LAB strains adapted to the Japanese climate and natural conditions for silage-making.

In this paper, we summarize the modification of the pouch method with the identification of microorganisms (CFB and yeast) for the pouch method. We also summarize the results of culture profiles in the modified pouch method.

Materials and methods

1. Microorganisms

Typical silage microorganisms (LAB, yeast and coliform bacteria: CFB) used in this study are listed in Table 1 with their isolation origins.

2. Silage materials

Corn stalk residue (remaining plant material above ground after harvesting baby corn ears) and Napiergrass were tested for the pouch method as silage materials. Corn stalk residue and Napiergrass were collected in Nakhon Pathom Province and Pathom Thani Province, Thailand, respectively. Corn stalk residue was cut into about 2–3 cm lengths, dried at 80ºC for 3 to 4 days, powdered and autoclaved at 121ºC for 20 min. Napiergrass, cultivated for 30 days (moisture content 88.8%), was cut into about 2 cm lengths, dried at 80ºC for 2 days and autoclaved at 121ºC for 20 min (pH of suspension in distilled water 6.01).

After autoclaving, both materials were adjusted to a moisture content of 75% by adding distilled water with 1.5% glucose added(w/w). The resulting silage materials were used as the media of the experiments. The content of sugar (glucose, fructose and sucrose) utilizable by LAB in corn stalk residue and Napiergrass after adjusting the moisture content to 75% was about 0.6% and 0.05%, respectively.

3. Pouch method

The medium (40 g), inoculated with the designated number of silage microorganisms, was put into a plastic bag with a double film of nylon and polyethylene (Hiryu KN 210, Asahi-Kasei Co., Japan, 20 × 30 cm, 0.1 mm thickness, oxygen permeability 1 mL/m^2/day), sealed by a vacuum sealer machine and cultured for 6, 24 and 504 h (21 day), anaerobically. In consideration of outdoor silage-making with direct sunshine, culture temperature was set at 45°C. A strain of LAB was inoculated together with a strain of CFB and yeast. Strains tentatively identified as *Enterobacter* sp. SG1-1T and *Saccharomyces cerevisiae* SG2-1Y were used as CFB and yeast, respectively. All experiments were conducted in duplicate.

4. Enumeration method of microorganisms

Each silage sample (10 g) was suspended in 30 mL of sterilized saline solution to make a sample suspension for the enumeration of microorganisms. The numbers of microorganisms were determined by a plate culture count method using lactobacilli MRS broth agar (Difco, USA) for LAB, violet red bile agar with lactose (Difco, USA) for CFB and potato-dextrose agar (Nissui Seiyaku Ltd., Japan) for yeast, respectively. Each plate was cultured for 24 h at 45ºC in an anaerobic box for LAB, 37°C for CFB and 30ºC for yeast. Colonies were counted and their

Table 1. List of microorganisms used in this study

Strains	Origins
LAB: LG 2-1	Lactobacilli strain isolated from silage made from Guinea grass harvested in Lopburi Province with addition of glucose (2%, w/w) in our laboratory (pH 4.54, dry matter content 60.8%).
LAB: N-22	Lactococci strain isolated from fermented vegetable matter.
CFB: SG 1-1T	Isolated from silage made from Guinea grass harvested in Saraburi Province with addition of glucose (2%, w/w) in our laboratory (pH 4.54, dry matter content 65.0%). Tentatively assigned to *Enterobacter* sp.
Yeast: SG 2-1Y	Isolated from the same silage with the isolation of an CFB strain. Tentatively assigned to *Saccharomyces cerevisiae*.

numbers were expressed as visible numbers of microorganisms in colony-forming units (cfu) per gram of fresh matter. Data were obtained from double examinations.

Results and discussion

The original pouch method[6,7] was developed to screen LAB strains which were used as silage additives under Japanese climatic conditions. The pouch method used alfalfa hay cube powder and a plastic film pouch to represent silage material and silo, respectively. A strain of CFB and a strain of butyric acid bacteria (BAB) together with LAB were used as typical microorganisms of silage fermentation in Japan. By using this pouch method, the lactic acid productivity of LAB strains in conditions reflecting silage fermentation are quickly examined and their abilities as silage fermentation starters are easily evaluated. Therefore, we tried to modify the pouch method for evaluating LAB strains used in Thailand.

1. Identification of a CFB strain and a yeast strain used in the modified pouch method

It was already reported that CFB and yeast as well as LAB were typically dominant in silage prepared in Thailand[5]. Strains belonging to these 3 microbial groups (LAB, CFB and yeast instead of BAB) correspond to typical microbial groups used for the pouch method in Japan. Therefore, a CFB strain SG 1-1T and a yeast strain SG 2-1Y isolated from silage (see Table 1) were used in these experiments.

Strain SG 1-1T is a Gram-negative straight rod with peritrichous flagella which grows in the presence and absence of oxygen. This strain was able to grow on media containing peptone, meat extract or lactic acid as a carbon source but did not grow on medium containing 6.5% sodium chloride. Bergey's manual[2] assigns this strain to the genus *Enterobacter*. Further identification of this strain requires additional biochemical and genetic examinations.

Strain SG 2-1Y is a sub-global cell, typically with polar budding and forming 2–3 ascospores which have a smooth surface and sub-global shape. This strain has no ability to utilize lactose or nitrate. These properties clearly suggest that this strain belongs to the genus *Saccharomyces*. The results of gas production tests from various kinds of sugar and the growth at 30, 37 and 40ºC are summarized in Table 2. This strain was tentatively assigned to *Saccharomyces cerevisiae*[1]. However, the final determination requires the results of biochemical and genetic examinations.

2. Selection of silage material

In Thailand, various kinds of agro-waste and pasture grasses are used for silage materials. Among them, corn stalk residue and Napiergrass were examined for the modified pouch method because of their high nutrient content and ready availability.

The medium using corn stalk residue was unfavorable for growth of silage microorganisms because the medium initially had a pH of about 4.9. This low pH could have originated from lactic acid fermentation during the process of gathering, cutting and drying the corn stalk residue. For example, when the inoculum sizes of each strain were adjusted to the same level, the counts of each microorganism were almost the same (10^5 cfu/g) after 24 h culture. However, an inoculated microorganism occupied about 10^2–10^3 times higher population density than that of the other microorganisms when the strain was inoculated at a high population density (10^3 times higher). These results showed that a microorganism initially inoculated at a large population density dominated the microbial population in the silage (data not shown) and therefore corn stalk residue medium was unable to be used for the observation of microbial changes in silage fermentation.

Silage microorganisms in Napiergrass medium grew smoothly (data shown in the next section) and had unique

Table 2. Taxonomic properties of yeast strain SG 2-1Y

Items	SG 2-1Y	*Sacchalomyces cerevisae*
Utilization of N-source		
KNO_3	–	–
$(NH_4)_2SO_4$	+	+
Peptone	+	+
Gas production from		
Cellobiose	–	–
Galactose	+	V
Glucose	+	+
Lactose	–	–
Maltose	±	V
Melibiose	–	V
Raffinose	–	V
Sucrose	+	V
Trehalose	+	V
Sol. Starch	–	V
Growth at		
30ºC	+	+
37ºC	+	V
40ºC	+	V

+: positive, – : negative, V: varied.

growth properties. Further, it is easy to collect and prepare Napiergrass at almost the same growth. Therefore, Napiergrass (a common pasture grass in tropical regions) was used for further experiments.

3. Pure culture of typical silage microorganisms in Napiergrass medium

As shown in Table 3, strain LG 2-1 used as a lactobacilli model strain grew well in both anaerobic and aerobic conditions. The count of colonies remained at the same level at 6 and 24 h. However, its growth was repressed (moderate growth) in acidic medium made by adding lactic acid (pH less than 4). Strain N-22 used as a lactococci model strain showed good growth in both anaerobic and aerobic conditions (10^8 cfu/g within 6 h culture), but growth in acidic medium was moderate and similar to strain LG 2-1. These data show that strain N-22 has a large specific growth rate, and the count of colonies remained at high levels at 6 and 24 h in anaerobic medium.

On the other hand, CFB strain SG 1-1T showed good growth in both of anaerobic and aerobic conditions and the count of colonies remained at high levels. This strain unexpectedly showed moderate growth, rather better growth than LAB strains, in acidic medium. These data are important because strain SG 1-1T is a useful index strain for the silage fermentation model system to evaluate the ability of a LAB strain as a starter strain (additive). The LAB strain is evaluated as having a favorable character for silage-making if growth of CFB strain SG 1-1T in a mixed culture is repressed or inhibited by the LAB strain. Yeast strain SG 2-1Y showed good growth in all conditions tested as expected.

From these data, it was considered that Napiergrass medium had no problem for being used in the modified pouch method because typical silage microorganisms grew well in this medium.

4. Mixed culture of typical silage microorganisms in Napiergrass medium

The growth properties of strains LG 2-1 and N-22 (large specific growth rate) in pure culture described previously were also observed in mixed culture with CFB and yeast as shown in Fig. 1. However, the medium pH after 24 h culture (5.34 for LG 2-1 and 5.66 for N-22) remained above 5.0 in spite of microorganism counts of 10^8 cfu/g. The reason is not clear. The maximum population density in silage was that of LAB owing to the inoculum size of LAB which was 10^3 times higher than that of CFB and yeast. Therefore, the effect of inoculum size on the growth of each silage microorganism in mixed culture was examined. The counts of microorganisms after inoculating LAB strains LG 2-1 and N-22 are shown in Tables 4 and 5, respectively. When the inoculum size of the 3 microorganisms were the same level, the counts of each microorganism after 6 h and 24 h culture were almost the same. However, strain LG 2-1 occupied the maximum population density in silage at 24 h culture and slightly repressed the growth of the CFB strain when the strain was inoculated with a population density 10^3 times higher than CFB and yeast strains. On the other hand, strain N-22 occupied the maximum population density in

Table 3. Counts of silage microorganisms in pure culture using Napiergrass medium

Silage microorganisms	Aeration	Addition of lactate	Medium pH after		Count of microorganism (cfu/g)	
			6 h	24 h	6 h culture	24 h culture
LAB: LG 2-1	+	–	5.63	4.50	3.0×10^7	4.2×10^7
	–	–	5.55	4.64	7.8×10^7	8.4×10^7
	–	+	3.91	3.48	7.5×10^6	6.2×10^6
LAB: N-22	+	–	5.15	4.36	1.1×10^8	2.3×10^7
	–	–	5.08	4.63	1.0×10^8	1.1×10^8
	–	+	3.86	3.93	7.1×10^6	6.8×10^6
CFB: SG 1-1T	+	–	5.47	5.34	3.0×10^7	1.2×10^8
	–	–	5.46	5.33	3.9×10^7	1.1×10^8
	–	+	3.46	3.63	1.7×10^7	1.2×10^7
Yeast: SG 2-1Y	+	–	5.45	5.32	4.7×10^7	5.4×10^7
	–	–	5.43	5.32	5.4×10^7	7.2×10^7
	–	+	3.44	3.68	1.7×10^7	1.4×10^7

Medium used in this study consisted of about 2 cm lengths of cut Napiergrass (moisture content 75%) with 1.5% glucose (w/w) and/or 2% lactate (w/w). The medium was inoculated with a silage microorganism at inoculum size of 10^5 cfu/g then cultured at 45°C for 6 or 24 h, aerobically or anaerobically.

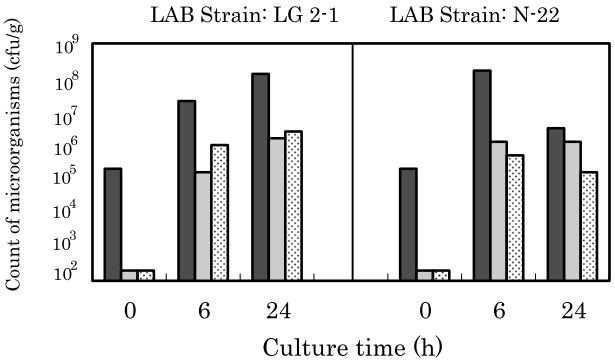

Fig. 1. Colony counts of typical silage microorganisms in mixed culture using Napiergrass medium

■ : LAB, □ : CFB, ⊠ : Yeast.

Medium used in this study consisted of about 2 cm lengths of cut Napiergrass (moisture content 75%) and 1.5% glucose (w/w). After inoculation of LAB (10^5 cfu/g), CFB (10^2 cfu/g) and yeast (10^2 cfu/g), the medium was cultured at 45°C for 6 or 24 h in an anaerobic jar.

silage at the 6 h stage of the fermentation. The count of LAB at 24 h was 10^2 times lower than that at 6 h when the inoculum size of the strain was 10^3 times higher than that of CFB and yeast. These results suggest that the inoculum size is an important factor in evaluating LAB strains using the modified pouch method. In addition, both LAB strains never showed a clear growth repression against the yeast strain in spite of LAB inoculum size 10^3 times higher than yeast. The count of yeast colonies in mixed culture was almost the same as that in pure culture.

5. Modified pouch method as a silage fermentation model system to evaluate LAB

By using Napiergrass as a silage material and *Enterobacter* sp. SG 1-1T and *Saccharomyces cerevisiae* SG 2-1Y as typical silage microorganisms as well as the LAB strain, the original pouch method[6,7] was modified to a silage fermentation model system adaptable to tropical conditions. In this modified pouch method, typical silage

Table 4. Effect of inoculum size on the growth of LAB strain LG 2-1, CFB and yeast in Napiergrass medium

Inoculum size (cfu/g)			Culture time (h)	Medium pH after 24 h	Count of microorganisms (cfu/g)		
LAB	CFB	Yeast			LAB	CFB	Yeast
10^2	10^2	10^2	6	5.69	1.1×10^6	1.2×10^6	1.1×10^6
			24	5.47	2.8×10^6	3.2×10^6	2.0×10^6
10^5	10^2	10^2	6	5.69	3.2×10^7	2.4×10^5	9.8×10^5
			24	5.43	1.2×10^8	2.2×10^6	4.0×10^6
10^2	10^5	10^2	6	5.86	1.1×10^6	2.8×10^7	1.0×10^6
			24	5.55	2.0×10^6	2.0×10^6	2.6×10^6
10^2	10^2	10^5	6	5.51	1.2×10^6	3.2×10^7	2.4×10^7
			24	5.52	2.8×10^6	1.0×10^8	1.5×10^8

Medium used in this study consisted of about 2 cm lengths of cut Napiergrass (moisture content 75%) with 1.5% glucose (w/w) and/or 2% lactate (w/w). The medium inoculated with LAB strain LG 2-1 together with CFB and yeast was cultured at 45ºC for 24 h, anaerobically.

Table 5. Effect of inoculum size on the growth of LAB strain N-22, CFB and yeast in Napiergrass medium

Inoculum size (cfu/g)			Culture time (h)	Medium pH after 24 h	Count of microorganisms (cfu/g)		
LAB	CFB	Yeast			LAB	CFB	Yeast
10^2	10^2	10^2	6	5.89	1.0×10^5	1.1×10^6	1.0×10^6
			24	5.59	1.0×10^5	9.0×10^5	1.0×10^5
10^5	10^2	10^2	6	5.79	1.9×10^8	1.3×10^6	6.8×10^5
			24	5.66	5.0×10^6	1.2×10^6	1.8×10^5
10^2	10^5	10^2	6	5.91	1.0×10^5	7.8×10^7	1.0×10^6
			24	5.44	1.0×10^6	8.0×10^5	2.0×10^5
10^2	10^2	10^5	6	5.77	4.4×10^7	6.0×10^7	1.4×10^7
			24	5.55	2.8×10^6	6.2×10^8	9.2×10^6

Medium used in this study consisted of about 2 cm lengths of cut Napiergrass (moisture content 75%) with 1.5% glucose (w/w) and/or 2% lactate (w/w). The medium inoculated with LAB strain N -22 together with CFB and yeast was cultured at 45ºC for 24 h, anaerobically.

microorganisms can grow well and especially LAB strains showed their unique growth properties in the mixed culture as shown in Tables 4 and 5. From these results, this modified pouch method seems to be useful for evaluating LAB strains as silage fermentation starter in Thailand and also tropical regions. Further, this method can be efficiently used for evaluating a large number of LAB strains because it is a quick and easy method. In addition, the screening of LAB strains to make good quality silage in Thailand by using the modified pouch method is reported in a separate paper.

Conclusion

1. The original pouch method was modified as a silage fermentation model system adaptable to tropical environments by using Napiergrass as a silage material and a strain of *Enterobacter* sp. SG 1-1T and *Saccharomyces cerevisiae* SG 2-1Y as CFB and yeast, respectively.
2. In the modified pouch method, typical silage microorganisms grew well and two LAB strains used as model strains showed their unique growth properties.
3. It was considered that this modified pouch method seems to be useful for the screening of LAB strains as silage fermentation starter in Thailand.
4. In addition, this method is able to quickly screen a large number of LAB strains because of its simplicity.

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