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Screening of Lactic Acid Bacteria Suitable for Silage-Making in Tropical Regions

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Abstract
Lactic acid bacteria suitable for silage-making under tropical conditions were screened based on experiment on liquid culture and model system for silage fermentation (pouch method). We selected a lactic acid bacterium strain I-7 from many isolates from silage. The strain belonging to the genus *Lactobacillus* showed a good adaptability to silage making at 45-52°C. Furthermore, the latic acid productivity (LAP) of the strain was inhibited by the addition of lactic acid to the liquid medium. However, the degree of inhibition was lower than that of other strains and remained a level of approximately 80 and 40% of that of the initial LAP in the presence of 0.5 and 1.0% lactic acid, respectively.

Discipline: Animal industry
Additional key words: thermotolerant strain, tropical silage

Introduction

Silage is an important cattle feed. The amount of silage produced in Japan increased rapidly, in particular during the last 10 years, and its rapid increase was promoted by the development of techniques to make high quality silage[1]. On the other hand in tropical regions, silage production is not a common practice for livestock farmers in spite of its importance as cattle feed, especially in the dry season[2,7], due mainly to the cost of silage-making and to the difficulty in making high quality silage depending on the adaptability of lactic acid bacteria (LAB). The LAB adapted to high temperatures should play an active role in ensilage. Therefore it is necessary to develop a good silage inoculant adapted to tropical conditions such as high temperature, high moisture level, high content of fiber, etc. However, research on tropical LAB inoculants has not been promoted.

We succeeded in isolating a LAB strain which showed a good adaptability to silage-making at 42°C[10]. Since the strain grows hardly at temperatures above 45°C, it is unlikely that it can be adapted to silage making in tropical regions. Therefore, we screened thermotolerant LAB strains to develop a new inoculant for making high quality silage in tropical regions as well as in the southwestern area of Japan.

In this paper, we summarized the results of screening of LAB in Thailand together with the lactic fermentation characteristics of some selected strains.

Materials and methods

(1) **Silage:** Corn silages, gathered in the Chiang Mai area in northern Thailand, were used in this study. Tropical grass silages which we prepared from

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a gramineous plant (*Paspalum notatum*), leguminous plant (*Leucaena leucocephala*) and a mixture of both plants in the laboratory in Bangkok, were also used in this study. Tropical grass silages were prepared by applying the pouch method[9,10]. The pouch which consists of a plastic bag with a double film of nylon and polyethylene (Hiryu KN210, Asahi-Kasei Co., Japan, 20 × 30 cm, 0.1 mm thickness) was packed with about 100 g of plant materials chopped into fragments 2 to 3 cm long to which 1% of glucose was added. Then, the pouches were heat-sealed after air-ejection using a vacuum packaging machine (National Hi-seal BH-950, Matsushita Denki Co., Japan) and were kept at 45°C for 4 or 5 days.

(2) **Medium:** The lactobacilli MRS broth (Difco, USA) to which 1.8% of agar and 0.8% of $CaCO_3$ were added was used to isolate LAB strains from silages. GPY-medium composed of glucose 0.5%, polypeptone (Wako Co., Japan) 0.1%, yeast extract (Difco, USA) 0.1%, $CH_3COONa \cdot 3H_2O$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $MnCl_2 \cdot 4H_2O$ 0.005%, tap water and pH 5.3 adjusted with acetic acid was used to screen the ability of isolates to produce lactic acid.

(3) **Screening:** LAB were isolated from corn silages and grass silages by an ordinary plate culture method using MRS agar medium containing $CaCO_3$. Colonies which were soluble in $CaCO_3$ and formed clear zones around their own colonies on the medium plate were isolated at random and used in this study.

Each strain was inoculated into 10 ml of GPY-medium in a test tube and incubated in an anaerobic jar (BBL Gas Pak, USA) at 37, 45 or 52°C for 20 h as primary screening. After incubation, the culture broth was centrifuged at 10,000 rpm for 3 min and the contents of lactic acid and glucose in the medium were analyzed by high performance liquid chromatography (HPLC), respectively.

(4) **Silage adaptability test:** The strains selected from primary screening were tested for their silage adaptability by a model system for silage fermentation using the pouch method[10] as secondary screening. In this case, 5 g of sterilized alfalfa hay cube powder medium (AHC-medium: moisture content 85%, sugar content 0.48%, buffering capacity 72 mg lactate/g·dry matter) was put into a pouch (17 × 4 cm), inoculated with butyric acid bacteria (*Clostridium butyricum* HA1; 10^5 cfu/g), coliform bacteria (*Klebsiella pneumoniae* G1; 10^7 cfu/g) and LAB test strain (10^6 cfu/g), and the pouch was incubated for 72 h at 37, 45 or 52°C. After the incubation, the contents of lactic, n-butyric and acetic acid produced in the pouch were analyzed by HPLC.

(5) **Inhibition test for lactic acid productivity (LAP) by the addition of lactic acid:** GPY-medium to which a certain amount of Na-lactate solution (pH 5.3) was initially added was used for the inhibition test by lactic acid. The culture and the analysis were the same as those described in the screening.

(6) **Extraction of organic acid:** Distilled water (3 or 6 times, v/w) was added into the pouch, shaken for 5 min and kept at 4°C overnight. Then, the mash was centrifuged at 15,000 rpm for 3 min. The supernatant thus obtained was used as a sample for analysis of organic acids and glucose by HPLC.

(7) **Analysis:** Organic acids produced were analyzed by HPLC (Nippon Bunko, BTB-post column system) as described in our previous paper[6]. Glucose remaining in the pouch was also analyzed by HPLC (Shimadzu, SCR-101H$^+$ column) as described in our previous paper[8].

Results

1) Primary screening

The ability of the 76 strains isolated at random from silages to produce lactic acid in GPY-medium was examined at 37, 45 and 52°C. The results for typical strains are shown in Table 1. LAB isolated from corn silage were hardly adapted to culture at higher temperatures such as 45-52°C (data not shown). However, some strains isolated from gramineous plant silage (designated as strain number I-series) and leguminous plant silage (designated as strain number M-series) were well adapted to culture at higher temperatures, and showed a higher LAP at 45-52°C than at 37°C.

2) Secondary screening

The silage adaptability of the 11 strains selected in the primary screening was tested by using the pouch method[9,10]. Table 2 shows the typical results of the secondary screening. At 45°C, all the strains inhibited butyric fermentation and produced lactic acid. However at 37°C, 8 strains (M-4, M-9, M-18, I-4, I-7, I-8, I-15 and K-6) were unable to inhibit butyric fermentation. On the other hand, 5 strains (M-4, M-15, I-12, I-13 and K-6) hardly grew at 52°C and did not produce lactic acid as well as acetic and butyric acid.

Table 1. LAP of selected strains in liquid medium

Strain no.	Culture temp. (37°C)			Culture temp. (45°C)			Culture temp. (52°C)		
	pH	LAP[a]	YLG[b]	pH	LAP[a]	YLG[b]	pH	LAP[a]	YLG[b]
M- 4	3.83	1.76	100	3.80	1.79	96.23	4.53	0.59	86.76
M- 9	3.90	1.76	89.34	3.87	1.86	99.47	4.55	0.62	100
M-15	3.81	1.23	73.21	3.80	1.20	81.63	4.48	0.47	87.03
M-18	4.22	0.63	64.29	3.76	1.28	75.29	3.86	1.20	78.95
I - 4	3.89	1.40	100	3.70	1.62	100	3.91	1.13	100
I - 7	3.87	1.23	83.11	3.59	1.85	90.69	3.92	1.16	99.15
I - 8	4.14	0.84	84.00	3.57	1.88	85.07	3.81	1.48	100
I -12	3.87	2.48	69.08	3.72	2.55	84.16	4.45	0.77	63.11
I -13	3.68	2.79	82.30	3.71	2.67	83.18	4.51	0.70	79.55
I -15	3.95	1.87	76.02	3.98	1.85	91.13	3.98	1.41	78.77
K- 6	3.25	2.91	98.98	3.43	2.15	100	5.10	0.30	42.86

a): Amount of lactic acid produced (mg, from 4 mg of glucose).
b): Yield of lactic acid from glucose (%).

Table 2. Effect of culture temperature on silage fermentation using a pouch system for selected typical LAB

Strain no.	Culture temp. (37°C)				Culture temp. (45°C)				Culture temp. (52°C)			
	pH	Organic acid[a] (%)			pH	Organic acid[a] (%)			pH	Organic acid[a] (%)		
		L	A	B		L	A	B		L	A	B
M- 9	6.00	0	0.178	0.117	5.02	0.341	0.103	0	4.95	0.309	0.065	0
M-15	5.20	0.164	0.222	0	4.95	0.333	0.089	0	5.60	0	0	0
I - 7	6.00	0	0.245	0.110	5.30	0.262	0.069	0	4.90	0.336	0.038	0
I - 8	5.95	0	0.254	0.086	5.20	0.276	0.089	0	4.90	0.360	0.031	0
I -13	5.20	0.169	0.222	0	4.95	0.337	0.088	0	5.60	0	0	0
I -15	5.80	0	0.263	0.122	4.95	0.335	0.092	0	5.05	0.340	0.070	0

a): L; Lactic acid, A; Acetic acid, B; n-Butyric acid (Fresh matter basis).

Table 3. Brief identification (genus) of selected strains

Strain no.	Form	Gram strain	Catalase prod.	Gas prod.	TLF[a]	Putative genus	CC no.[b]	
							NGRI	TISTR
M- 4	Coccus (tetrad)	+	−	−	Homo	*Pediococcus*	2004	1020
M- 9	Coccus (tetrad)	+	−	−	Homo	*Pediococcus*	2009	1021
M-15	Coccus (tetrad)	+	−	−	Homo	*Pediococcus*	2015	1022
M-18	Long Rod	+	−	−	Homo	*Lactobacillus*	2018	1023
I - 4	Rod	+	−	−	Homo	*Lactobacillus*	1004	1024
I - 7	Rod	+	−	−	Homo	*Lactobacillus*	1007	1025
I - 8	Rod	+	−	−	Homo	*Lactobacillus*	1008	1026
I -12	Coccus(tetrad)	+	−	−	Homo	*Pediococcus*	1012	1027
I -13	Coccus (tetrad)	+	−	−	Homo	*Pediococcus*	1013	1028
I -15	Rod	+	−	−	Homo	*Lactobacillus*	1015	1029
K- 6	Coccus (strepto)	+	−	−	Homo	*Streptococcus*	3006	1030

a): Type of lactic acid fermentation.
b): Each strain was deposited in the culture collection of National Grassland Research Institute (NGRI), Nishinasuno, and of Thailand Institute of Scientific and Technological Research (TISTR), Bangkok.

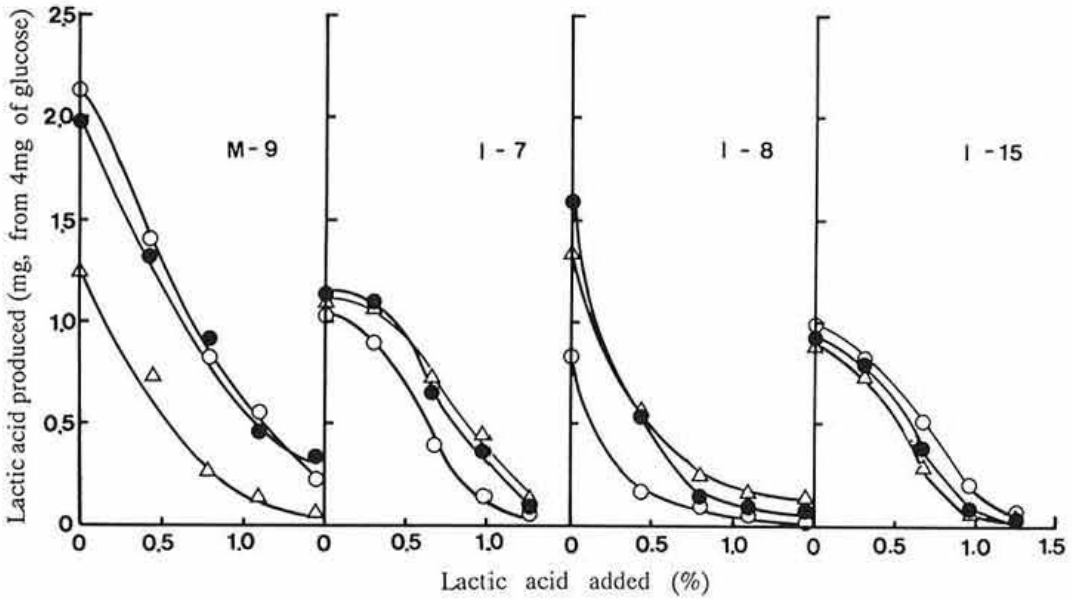

Fig. 1. Effect of culture temperature and lactic acid addition on the production of lactic acid by selected strains
Culture temperature: ○; 37°C, ●; 45°C, △; 52°C.
Each strain was cultivated in liquid medium to which a certain amount of lactic acid had been added at 37, 45 or 52°C for 20 h, anaerobically.

3) Inhibition test by the addition of lactic acid

The LAP of 4 strains selected from the secondary screening was tested at 37, 45 or 52°C in the GPY-medium to which a certain amount of lactic acid was initially added. Strain M-9 showed a higher LAP than other strains in a liquid medium to which lactic acid had not been added as shown in Fig. 1. The LAP of strains M-9 and I-8 was rapidly inhibited by a small amount of lactic acid, but the inhibition profiles were different from those of strains I-7 and I-15. The relative LAP in the presence of 0.5 and 1.0% lactic acid against initial production is shown in Fig. 2. Strain I-7 was resistant to the inhibition and more than 80% of LAP remained in the presence of 0.5% lactic acid compared with the absence of lactic acid. The LAP of strain M-9 and I-15 decreased when the culture temperature increased to 52°C, while in the case of strains I-7 and I-8, the LAP increased with the increase of the temperature.

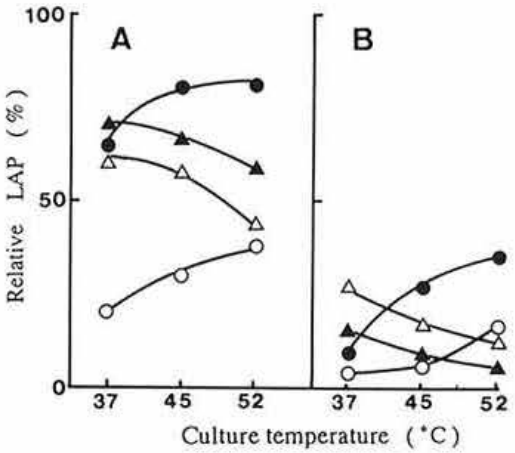

Fig. 2. Effect of culture temperature on the relative LAP in the liquid medium after the addition of 0.5% (A) and 1.0% (B) lactic acid
Strains: ●; I-7, ○; I-8, ▲; I-15, △; M-9.
Relative LAP was calculated from the LAP obtained in the presence of 0.5% lactic acid (A) or 1.0% lactic acid (B) against LAP obtained in the absence of lactic acid.

4) Identification of selected strains at generic level

The generic name of the selected strains was determined based on a manual[12]. From the results of Gram stain, catalase productivity, gas production from glucose, lactic acid yield and microscopic observation, these strains were identical with the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* as shown in Table 3. Among them, the genus

Lactobacillus and the genus *Pediococcus* were dominant in I-series and M-series, respectively.

Discussion

Silage is an animal feed that can be stored and its preservation properties depend on the pH which decreases by lactic fermentation in the ensiling process. Accordingly, the organic acid content is one of the important indices[3] for evaluating the silage quality. The quality of silage with a high lactic acid content and lacking butyric acid is considered to be high. To obtain silage with a high quality, LAB inoculants have been marketed. However, it is unlikely that marketable inoculants can be used in the tropical regions.

Therefore, we screened LAB for developing a silage inoculant adapted to the tropical conditions. As a result of primary and secondary screenings (Tables 1 and 2), strains M-9, I-7, I-8 and I-15 were selected as thermotolerant strains of silage LAB.

Among them, strain M-9 belonging to the genus *Pediococcus* showed a higher LAP than other strains in liquid medium without lactic acid at 37-45°C and LAP was strongly inhibited by the addition of lactic acid (Fig. 1). The high LAP of the strain is associated with rapid growth at the early stage of culture and the sensitivity to the inhibition may be typical of the genus *Pediococcus*. It is generally recognized that strains belonging to the genus *Pediococcus* predominate at the early stages of ensilage and are characterized by a short lag phase, a rapid rate of acid production and sensitivity to pH below 5[4].

On the other hand, strain I-7 belonging to the genus *Lactobacillus* did not exhibit a high LAP in the liquid medium lacking lactic acid, but was resistant to the inhibition by the addition of lactic acid (Fig. 1). The LAP level of strain I-7 at 52°C was approximately 80 and 40% of that of the initial LAP in the presence of 0.5 and 1.0% lactic acid, respectively (Fig. 2). The inhibitory characteristics of strains I-8 and I-15 resembled those of strains I-7 and M-9 (Fig. 2).

It is well known that the LAP of LAB is inhibited by lactic acid production in a non-competitive way[5]. In silage fermentation, it is important that the pH of silage should decrease to less than 4.2 by the effective production of lactic acid from sugar in a silo. Therefore, the tolerance to lactic acid is an important characteristic for the inoculants.

Based on these results, strain I-7 was selected for silage making. To use the strain as silage inoculant, however, additional ability should be met such as rapid growth at early stage of culture because its LAP is relatively low. It is considered that the strain could become a good material for silage inoculant.

It has been reported[11] that LAB belonging to the genera *Lactobacillus* and *Pediococcus* predominated in tropical silages. In our material, most of the selected strains belonged to both genera, although 75% of the strains of M-series isolated from leguminous plant silage belonged to the genus *Pediococcus* and this distribution is not in agreement with the report[11]. The discrepancy may be due to the fact that the strains of M-series were selected as thermotolerant strains though primary screening and did not exhibit the characteristics of the natural microbial flora of silage LAB.

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