

## LACTIC ACID BACTERIA AND ENZYME PRODUCTION IN SILAGE OF GUINEA GRASS (*PANICUM MAXIMUM*)

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### Abstract

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Two different isolates of lactic acid bacteria, *Lactobacillus plantarum* H5-M13F and *Pediococcus pentosaceus* Ac2-M13F were confirmed by morphological and phylogenetic identification. H5-M13F was rod shaped, gram positive, negative for gas production and catalase activity, homofermentative, and grew at 10 – 45 C, pH 3.5 – 9.6, and 1.0 – 10 % NaCl<sub>2</sub>. Isolate Ac2-M13F was coccus shaped, gram positive, negative for gas production and catalase activity, homofermentative, and grew at 10 – 45 C, pH 3.5 – 9.6, and 1.0 – 6.5 % NaCl<sub>2</sub>. Amplification of the ITS regions from isolates H5-M13F and Ac2-M13F were confirmed by using Primer OPA-3. The phylogenetic tree from PAUP analysis indicated that isolate H5-M13F clustered with *Lactobacillus* sp. and isolate Ac2-M13F with *Pediococcus* sp. Phylogenetic analysis and dendrograms revealed no relationship between the two groups. It was found that isolate H5-M13F mostly isolated from silages (87.59%) while isolate Ac2-M13F was found at a low incidence in silages (3.19%). *Lactobacillus plantarum* H5-M13F and *Pediococcus pentosaceus* Ac2-M13F from silage of guinea grass showed the ability to produce extracellular degradative enzymes. *Pediococcus pentosaceus* Ac2-M13F produced amylase and protease and *Lactobacillus plantarum* H5-M13F produced protease.

*Key words*: lactic acid bacteria; enzyme; silage; guinea grass

### Introduction

Guinea grass (*Panicum maximum*) is a fiber resource generally used for ruminant feed in Thailand (Aganga and Tshwe-nyane, 2004). One problem is the lack of subsequent crops of good quality roughage which can be fed to dairy cows during the dry season. Therefore, implementation of quality roughage in the rainy season comes to storage in the form of fermented forage or silage that can solve the feed shortage problem. The main factors involved are to produce silage more rapidly by using potent degradative microorganism especially lactic acid bacteria (LAB) (McDonald et al., 1991; Brookes and Buckle, 1992). The species diversity of bacteria may vary according to type of forage to be fermented (Cai et al., 1999a, 1999b; En-

nahar et al., 2003; Wang et al., 2006; Parvin and Nishino, 2009, 2010). This research was focused on finding potent LAB to be used in silage for animal feed. Enzyme production activities in silage are also available to break down forage fiber. Wallace et al. (2001) reported that the relationship between enzyme activities and *in vitro* gas production using grass and corn silage had significant positive correlations with cellulase activity and gas production from grass silage. The question is: how to improve the nutrition of farmers' ruminant animals when each animal raiser keeps only one animal? In flood or summer, the major fodders available are grass or rice straw, together with limited quantities of hay and concentrated feeds. As a minimum, it is essential to provide a green fodder supplement to enhance rumen function for bovine animals. For animal raisers with lim-

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ited production capacities, finding enough feed in the flood or summer months to maintain good production is always a problem. There are many forced to buy roughage, concentrates or silage just to keep their animals alive and are unable to benefit financially due to the higher prices paid for animal feed in the flood or summer months. The objective of this research was to identify and investigate effective LAB producing enzymes to degrade silage consisting of guinea grass (*Panicum maximum*) for animal feed.

## Materials and Methods

### Isolation and identification of lactic acid bacteria from silage

#### Silage preparation

Forty-five day-old guinea grass (*Panicum maximum*) was cut into 5 cm pieces by sickle starting at the ground level. The harvested material samples were chopped to 2-3 cm by knife. The pre-silage material samples were mixed with 1% NaCl<sub>2</sub>, put in polyethylene bags and stored at ambient temperature for 21 days. A total of 25 g per samples was dissolved in 100 ml sterile water and stirred for 10 min. The pH values were measured using a pH meter (Polan et al., 1998). After a 21-day fermentation, the color and aroma of the silages were described according to the score indices of Muhammad et al. (2008). For the color description, the silage was scored as 1 = dark or deep brown, 2 = light brown, 3 = pale yellow and 4 = yellowish green. For the aroma description, the silage was scored as 1 = putrid or rancid, 2 = pleasant, 3 = sweet and 4 = very sweet. The analysis of total count of lactic acid bacteria was done by dilution plate method.

#### Isolation

The samples were collected from the silage in three parts of each silo, upper, middle and lower parts. The samples consisted of 100 g from each part which were mixed and 10 g randomly collected sub samples were diluted with distilled water then mixed with 90 ml of 0.1 % peptone. The sample was serially diluted up to 10<sup>-6</sup>, then 0.1 ml was pipetted and spread plated on de Man, Rogosa and Sharpe broth (MRS) mixed with calcium carbonate 1 % (de Man et al., 1960) and incubated at 30 ± 4°C for 48 h. The single colonies observed and isolated into pure culture.

#### Morphological identification

Gram stain technique was used to differentiate two large groups of bacteria between Gram positive and Gram negative which appeared violet or red, respectively. Those bacteria that hold on to the primary iodine dye complex and remain violet were characterized as called Gram positive and those which are decolorized and subsequently take up the counterstain (pink/

red) that were characterized as gram negative. The morphology of isolated LAB were noted based on the methods of Kandler and Weiss (1986) (Gram's stain, Catalase test, and Gas production test). The resultant data were compared with the descriptions of Kandler and Weiss (1986) and Stiles and Holzappel (1997). The catalase production test was done using a dropper or Pasteur pipette, placing 1 drop of 3% hydrogen peroxide onto the microorganism on the microscope slide, which were mixed. The microscope slide was immediately covered with a lid to limit aerosols and observe for immediate bubble formation (O<sub>2</sub> + water = bubbles). The formation of bubbles against a dark background enhanced readability. Positive reactions were indicated by immediate effervescence (bubble formation). The microscope slide placed over a dark background viewed with a magnifying glass or microscope (40x) was used to observe weak positive reactions. No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction (South Bend Medical Foundation, 2010). Gas production was tested by transferring pure cultures into MRS mixed with 1% NaCl<sub>2</sub>. Durham tubes containing sterilized liquid media were incubated at 30 ± 4°C for 48 h. Tubes filled with gas, indicated heterofermentation, tubes without gas, homofermentation (Hayward, 1957). A growth test with different temperature regimes was done by transferring pure cultures into sterilized MRS media and incubating at 10, 30 and 45°C for 48 h, then observing for turbidity (Cai et al., 1998; Yang et al., 2010). Salt tolerance was tested at different concentrations of NaCl<sub>2</sub> by transferring pure cultures into sterilized MRS mixed with sterilized NaCl<sub>2</sub> at 1, 3, 6.5 and 10% w/v, and incubating at 30 ± 4°C for 48 h and observing for turbidity (Cai et al., 1998; Yang et al., 2010). The growth test for different pH levels was done by transferring pure cultures into sterilized MRS with pH adjusted to 3.5, 5.7 and 9.6, incubating at 30 ± 4°C for 48 h and observing for turbidity (Kandler and Weiss, 1986; Yang et al., 2010). Morphological identification was done by following the methods of the Bergey Manual of Determinative Bacteriology (1994).

#### Phylogenetic identification

The isolates of LAB were separately cultured on MRS mixed 1% calcium carbonate, incubated at room temperature (30 ± 4°C) for 24-48 h; single colonies were collected for DNA extraction and observed under a compound microscope for morphological characteristics (Oneca et al., 2003). The OPA-3 primer (5'-AGTCAGCCAC-3') was used (Quere et al., 1997). The 25 µl PCR solution consisted of 1X PCR buffer with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Tris-HCl 75 mM, pH 8.8, 25°C, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 20 mM and Tween 20 0.01%), MgCl<sub>2</sub> 2 mM, dNTP 400 µM, primer OPA-3 0.4 µM, Taq DNA polymerase 0.1 unit/ µl and the cultured colony to amplify DNA fragments. A cycle was consisted

of an initial denaturation at 94°C for 5 min and followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 36°C for 1 min and extension at 72°C for 1 min. PCR products were sent to purify and sequence. The sequences were aligned and adjusted using Clustal X program for phylogenetic analysis. DNA sequences were edited and aligned with the BioEdit program, version 7.0.5 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Sequences were multiply aligned with Clustal X version 1.83 before performing the analysis using the maximum parsimony method with (PAUP) 4.0b (Swofford, 1998). For maximum parsimony analyses, bootstraps of 1000 replicates were performed to examine the relationships of each isolate. Maximum parsimony trees were calculated via fast stepwise addition with representative isolates from GenBank (<http://www.ncbi.nlm.nih.gov>) and *Enterococcus* spp. was used to analyze as the out-group. Genetic relationships among *Lactobacillus* spp. and *Pediococcus* spp. isolates were determined using the sequences of the ITS region of rDNA to construct phylogenetic trees.

#### Proximate composition analysis of silage

For each treatment, 1000 g of fresh material were randomly collected to determine nutrient composition. The samples were oven dried at 60°C for 48 h prior to proximate analysis. Dry matter (DM), Ash, Crude fat (CF), Ether extract (EE), Crude protein (CP) and Crude fiber (CF) were determined according to the methods of AOAC (1995). Neutral detergent fiber (NDF) and Acid detergent fiber (ADF) were determined according to the method of Van Soest and Robertson (1979). All analyses were conducted using the Fibertec System M6 (FOSS, USA).

#### Enzyme production

The two species of LAB isolated from silage were selected for their ability to produce extra cellular degradative enzymes such as amylase, protease, lipase, cellulase and ligninase. Three replicates of each treatment were assayed and non-transferred plates served as negative controls. Transferred plates were incubated at 25°C, and checked at either 5 or 10 days depending on the growth rates. When the colonies grew over 50-60% of the area of the plate, chemical indicators were added to assay enzyme activity and activity zones.

**Amylase** – A drop of the bacterial culture was streaked onto a starch agar plate, then incubated at 30°C for 48 h. When colonies were visible, the plate was flooded with Lugol's solution, then the clear zone surrounding the colony was monitored. If the starch was hydrolyzed by the excreted amylase, a clear zone around the bacterial colony appeared. A yellow zone around the colony in an otherwise blue medium was considered a positive test for starch hydrolysis as modified by Gessner (1980).

**Protease** – Skim milk agar plates were streaked with bacterial colonies, then incubated at 30°C for 48 h, and the clear zone

surrounding the colony was monitored. Clear zones surrounding visible colonies implies a positive caseinase (protease) reaction (Folasade and Joshua, 2005).

**Lipase** – Lipase activity test was performed by growing the isolates on trypticase soy agar. A volume of 3.5 ml of melted fat stained with Nile blue sulfate was added to 70 ml of trypticase soy agar medium the plates were streaked with pure cultures and one non-inoculated plate served as the negative control. A positive test was indicated by the occurrence of precipitated fatty acid crystals around the colony (Abdel-Rheem and Shearer, 2002).

**Cellulase** – Cellulase activity was assessed using cellulose azure agar. The tested LAB were assayed by growing on Peptone Yeast Glucose (PYG) medium which consisted of peptone 1.25 g, yeast extract 1.25 g, glucose 3 g, agar 18 g in 1000 ml of sterilized water. Thereafter, the bacteria transferred to Cellulolytic Basal Medium (CBM) which consisted of NH<sub>4</sub>H<sub>2</sub>P<sub>0</sub> 0.1g, KCl 0.2g, CaCl<sub>2</sub> 0.2g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2g, 4% of CMC 250 ml, agar 18g in 750 ml of sterilized water. The plates were incubation for 7-10 d before observation for the colony grow over 50% of the Petri dish. Then 2% of congo red solution was poured on the control and treated plates, and after 15 min was washed by sterilized water. Then 1M NaCl<sub>2</sub> solution was added and after 15 min a clear zone surrounding the colony was monitored. If a clear zone appeared, it implied to the production of cellulase (Paterson and Bridge, 1994).

**Ligninase** – Ligninase activity was determined by the peroxidase test in PYG and then transferred to corn meal agar (CMA) which consisted of corn meal agar 20 g per 1000 ml and incubated for 5-10 d. The colonies were observed and then using a sterilized cork borer (0.5 cm diameter) wells were made at the growing edges of colonies. One drop of solution was dropped into a well which consisted of 1% w/v of perogallic acid and 4% hydrogen peroxide. A positive test was indicated by the formation of a golden yellow to brown color around the colonies (Abdel-Rheem and Shearer, 2002).

## Results and Discussion

#### Silage preparation

After 21 d of ensilage, the plastic containers were opened and examined for gross characteristics. Overall, the silages were of good color, aromatic and acidic. The silages contained an average of 76.47% moisture. The pH values of the silages averaged 5.53. The pH values indicated the anaerobic fermentation of the silages similar to the work of Schroeder (2004).

#### Isolation and Identification of lactic acid bacteria

##### Isolation

The colonies of LAB grown on MRS had different char-

acteristics and clear zones were observed around colonies due to the production of lactic acid which causes the conversion of calcium carbonate to calcium lactate (Frazier and Rupp, 1928). The colonies of LAB were white, opaque around colony circular and entire, tops of colonies were flat, raised, convex or umbonate. These characteristics conformed to the general characteristics of LAB (Kandler and Weiss, 1986). The average total count of LAB after fermentation was  $5.99 \times 10^6$  cfu/g.

### Morphological identification

- The morphology of the isolated lactic acid bacteria after staining is shown in Figure 1. All bacteria isolated were Gram positive and rod or cocci shaped. The tested bacteria were unable to produced catalase as indicated by the catalase enzyme test (Caplice and Fitzgerald, 1999) and were determined to be LAB. The gas production test for fermentation pathway in each of the samples found that the LAB were homofermentative. The samples of homofermentative were mostly rod shaped the rest were cocci.

- They were able to grow at 10, 30 and 45 degree Celsius, at NaCl concentrations of 1, 3, 6.5 and 10% and a pH of 3.5, 5.7 and 9.6 in each isolate that could be separated into 4 groups (A-D) as shown in Table 1. When compared with the characteristics described by Kandler and Weiss (1986) and Stiles and Holzapfel (1997). The 57 different isolates could be divided into two genera. *Lactobacillus* sp.H5-M13F was commonly found in silages (an incidence of 87.59%) Ennahar et al. (2003), Parvin and Nishino (2009) reported that this bacterium can be commonly detected in silage. *Pediococcus* sp. Ac2-M13F was found in silages at a lower incidence (3.19%).

### Phylogenetic Identification

Amplification of the ITS regions from isolates of *Lactobacillus* spp. and *Pediococcus* spp. used the Primer OPA-3. The phylogenetic tree from PAUP analysis placed the *Lactobacillus* spp. and *Pediococcus* spp. into two distinct groups. Phyloge-

**Table 1**

**The characteristics of lactic acid bacteria isolated from silage**

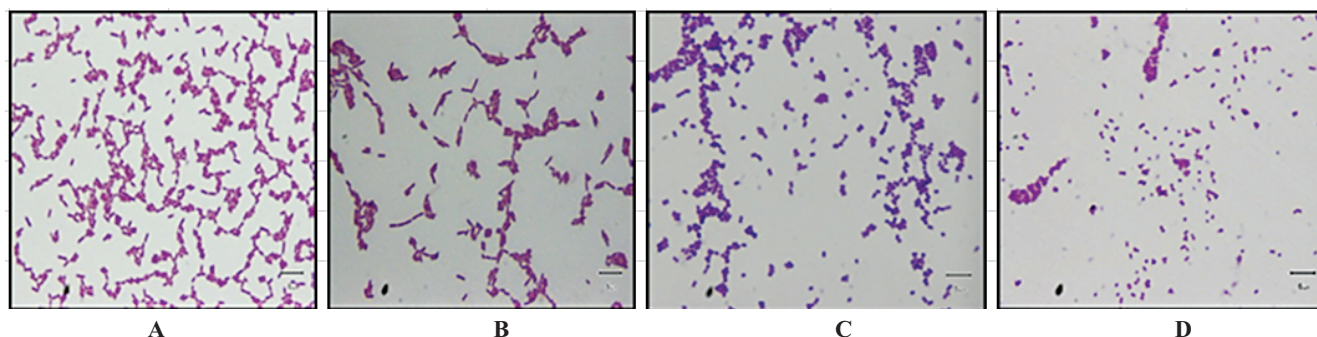
Characteristic	Group			
	A	B	C	D
Morphology	Rods	Rods	Cocci	Cocci
Gram	+	+	+	+
Catalase test	-	-	-	-
Gas test	-	-	-	-
Fermentation	Homo	Homo	Homo	homo
Temp				
10°C	+	+	-	-
30°C	+	+	+	+
45°C	+	+	+	+
pH				
3,5	+	+	+	+
5,7	+	+	+	+
9,6	+	+	+	+
NaCl				
1,00%	+	+	+	+
3,00%	+	+	+	+
6,50%	+	+	+	+
10,00%	±	-	-	-
Identified as	<i>Lactobacillus</i> sp.	<i>Lactobacillus</i> sp.	<i>Pediococcus</i> sp.	<i>Pediococcus</i> sp.

Note: + = Positive reaction, - = Negative reaction, ± = Variable reaction; Homo = Homofermentive

netic analysis produced a dendrogram indicating that there was no relationship between the *Lactobacillus* spp. and *Pediococcus* spp. groups (Figure 2).

### Proximate analysis of silages

The proximate composition of the examined silages (Table 2) showed that dry matter changed from 20.9% before treatment to 23.5% after treatment. Ash content was 15.7 %, crude protein 7.9%, crude fiber 42.6%, ether extract 4.2%, nitrogen free extract 22.6%, organic matter 84.2%, neutral detergent fiber 56.5% and acid detergent fiber 37.8%. Jolaosho et al. (2013)



**Fig. 1. Morphology of lactic acid bacteria isolated from silage**  
A and B showed characteristics of *Lactobacilli*  
C and D showed characteristics of *Pediococci*

**Table 2**  
**Composition of Guinea grass silage from Proximate analysis**

Composition	Before fermentation	After fermentation
Dry matter (DM)	20.9	23.5
Ash	11.1	15.7
Curde protein (CP)	7.9	7.9
Crude fiber (CF)	33.8	42.6
Ether extract (EE)	5.6	4.2
Nitrogen free extract (NFE)	35.9	22.6
Organic matter (OM)	88.8	84.2
Nutral detergent fiber (NDF)	58.6	56.5
Acid detergent fiber (ADF)	31	37.8

reported that the proximate composition and fiber fractions of silage from Guinea grass showed that DM was 21.03%, CP 9.48%, EE 6.5%, ASH 8.13%, NFE 15.58% NDF 60.31% and ADF 40.98%.

#### Enzyme production properties

The efficacy of *Lactobacillus* spp. and *Pediococcus* spp. from silage to produce extracellular degradative enzymes was examined. The results showed that the two genera were able to degrade, to some degree, starch and protein (Table 3). Lactic acid bacteria identified as *Pediococcus* sp. had able to produced amylase and protease. Iuchi et al, 2012 isolated *Pediococcus ethanolidurans* A4-27 from Japanese pickles (nuka-zuke) and found that it could produce a high molecular weight amylase. They indicated that the amylase enzyme from *P. ethanolidurans* was useful for fermentation of rice bran-bed (nuka-doko) which contained more than 15% NaCl.

*Lactobacillus* sp. produced protease indicated by clear zones around and below colonies.

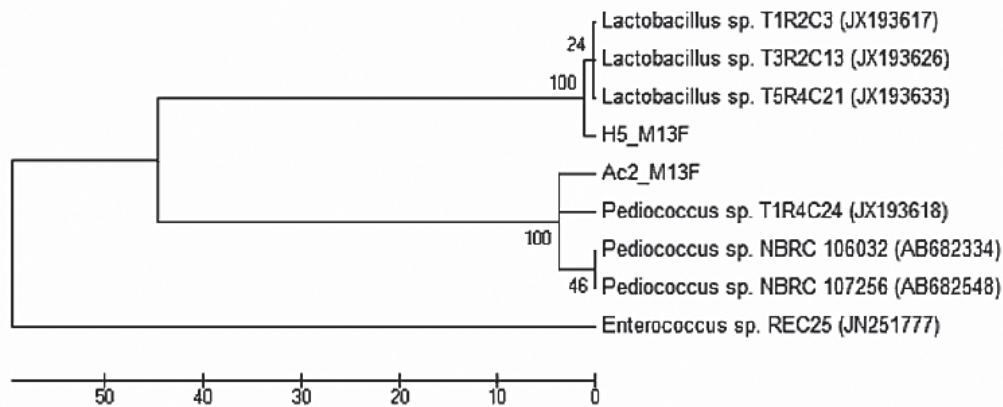
*Pediococcus* sp. produced amylase indicated by a yellow clear zone around the bacteria colonies.

#### Conclusion

Isolates of LAB were identified which based on their morphology were divided into two genera. *Lactobacillus* sp. H5-M13F was found at a high incidence in silages (87.59%) and *Pediococcus* sp. Ac2-M13F was found at a low incidence (3.19%). The phylogenetic tree from PAUP analysis placed the recovered *Lactobacillus* sp. and *Pediococcus* sp. into two distinct groups. Phylogenetic analysis revealed that there was no relationship between of the *Lactobacillus* sp. and *Pediococcus* sp. groups. The proximate composition of the examined silages showed that silages contained an average of 76.47% moisture. The pH values of the silages averaged 5.53, DM 23.5%, Ash content 15.7%, CP 7.9%, CF 42.6%, EE 4.2%, NFE 22.6%, OM 84.2%, NDF 56.5% and ADF 37.8%. *Lactobacillus* sp. could produce protease and *Pediococcus* sp. was able to produce amylase and protease. These two species can possibly be used to improve the nutritional value of the silage, TMR or roughage for ruminant production.

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**Fig. 2. Phylogenetic relationships of *Lactobacillus* spp. and *Pediococcus* spp. isolated, based on the ITS regions of rDNA sequences**

The phylogenetic tree was obtained by the maximum parsimony method using the PAUP4.0b program. *Enterococcus* sp. was used as the out group. The numbers above the lines represent the 1000 replicates parsimony bootstrap values

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