Isolation of Lactic Acid Bacteria from Post-Harvest Pineapple Plant Silage and Screening for Acid Production and Antifungal Activity against *Aspergillus flavus*

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ABSTRACT

Post-harvest pineapple plant residues are an abundant agricultural by-product in Thailand with potential for use as silage. This study aimed to isolate and screen indigenous lactic acid bacteria (LAB) from post-harvest pineapple plant silage for acid-producing and antifungal properties. Changes in pH and LAB populations during 21 days of natural ensiling were also investigated. The silage pH remained below 5.0 throughout ensiling, while the LAB population fluctuated during the early stage, decreasing from 9.98 log CFU/g at day 0 to 7.80 log CFU/g at day 21. A total of 300 presumptive LAB isolates were obtained. Of these, 196 isolates (65%) showed an acid-producing ability ratio >2.00 and were further screened against *Aspergillus flavus*. All 196 isolates inhibited fungal growth by more than 20%, and 90 isolates showed more than 35% inhibition. The final culture pH of these 90 isolates after 48 h in MRS broth ranged from 3.68 to 3.50. These results indicate that post-harvest pineapple plant silage is a source of LAB with both acid-producing and antifungal properties. Further screening and characterization are required to select promising strains for use as silage starter cultures.

Keywords: Lactic acid bacteria/ Post-harvest pineapple plant residues/ Silage/ *Aspergillus flavus*/ Antifungal activity

1. INTRODUCTION

Roughage is the main feed source for ruminants and is an important factor in livestock production, especially for beef and dairy cattle [14]. Roughage can be obtained from forage crops such as corn and Napier grass, as well as agricultural by-products such as pineapple stems and peels and mature leucaena stems [11]. However, forage production is not consistent throughout the year. During the rainy season, forage grows well and the amount produced may exceed livestock requirements, while forage production is limited during the dry season. Therefore, silage production is commonly used to preserve forage and provide feed during periods of shortage [4].

In tropical countries such as Thailand, silage production is important for maintaining feed availability during seasonal forage shortages [10]. However, natural fermentation without selected inoculants may result in losses due to undesirable microorganisms. Fungal contamination is also an important problem in silage. *Aspergillus flavus* is one of the important fungi because it can produce aflatoxins, which can affect animal health and productivity [3, 13]. The use of lactic acid bacteria (LAB) as starter cultures is one approach to improve silage fermentation. Some LAB, such as *Lactobacillus buchneri*, can promote acidification during ensiling and improve fermentation efficiency [7]. In addition, some LAB strains, including *Lactobacillus plantarum* and *Lactobacillus brevis*, have been reported to have antifungal or anti-aflatoxigenic activity against toxigenic fungi such as *A. flavus* [9].

Pineapple is an important industrial crop in Thailand and produces a large amount of post-harvest plant residues. Pineapple leaves have been estimated at approximately 37.5 tons per hectare, resulting in nearly 2.7 million tons of leaf waste annually in Thailand [1]. These residues are commonly managed by applying herbicides to terminate plant growth, followed by burning or incorporation into the soil. These practices may prolong land preparation, leave chemical residues in the soil, and contribute to environmental pollution [6] Therefore, post-harvest pineapple plant residues could serve as an alternative feed source and help reduce agricultural waste. Although pineapple plant residues can be

used for silage production, natural fermentation without selected microbial inoculants may result in undesirable microbial growth and fungal spoilage [8].

However, information on the LAB naturally present in post-harvest pineapple plant residues during ensiling remains limited. LAB that can produce acid may be useful for silage fermentation, while LAB with antifungal activity may also help control fungal growth. Therefore, this study aimed to isolate and screen acid-producing LAB with antifungal activity against *A. flavus* from post-harvest pineapple plant silage. Changes in pH and LAB populations during natural ensiling were also investigated.

2. METHODOLOGY

2.1 Preparation of fresh and ensiled pineapple plant residues

Pineapple plants were collected after fruit harvest and chopped into 3–5 cm pieces using a chopper machine. One kilogram of chopped material was tightly packed into double-layered plastic bags, and the air was manually expelled. The bags were stored at ambient temperature for 21 days. Samples were collected on days 0, 3, 5, 7, 14, and 21 of ensiling to determine LAB populations and pH.

2.2 Enumeration and isolation of lactic acid bacteria from fresh and ensiled pineapple plant residues

2.2.1 Enumeration of LAB populations

LAB populations were determined on days 0, 3, 5, 7, 14, and 21 of ensiling. A 50-g sample was mixed with 450 mL of 0.1% peptone solution and homogenized using a stomacher for 2 min. Serial decimal dilutions were prepared, and 0.1 mL of the appropriate dilution was spread on de Man, Rogosa and Sharpe (MRS) agar supplemented with $CaCO_3$. The plates were incubated anaerobically at 30°C. Colonies producing clear zones around the colonies were counted as presumptive LAB, and the results were expressed as colony-forming units (CFU).

2.2.2 Isolation and purification of LAB

Following enumeration, colonies producing clear zones on MRS agar supplemented with $CaCO_3$ were selected and purified. The isolates were presumptively identified as LAB based on Gram-positive staining, rod or coccal morphology, absence of spore formation, and a negative catalase reaction.

2.3 Screening and selection of LAB isolates

To select LAB isolates with high acid-producing ability, purified LAB isolates were screened on MRS agar supplemented with $CaCO_3$. Each LAB isolate was activated in MRS broth at 30°C for 48 h. An aliquot (10 µL) of each LAB culture was spot-inoculated onto MRS agar supplemented with $CaCO_3$ in triplicate. The plates were incubated anaerobically at 30°C for 48 h. The diameters of the colonies and clear zones were measured, and the acid-producing ability was calculated as follows:

$$\textbf{Acid-producing ability ratio} = A/B$$

Where A is the total diameter of the clear zone, including the colony, and B is the diameter of the LAB colony. Isolates with high acid-producing ability ratios were selected for further analysis.

2.3.1 Antagonistic activity against Aspergillus flavus

The LAB isolates selected for acid production were further screened for antagonistic activity against *A. flavus* M3T8R4G3 using the dual culture method [16].

Prior to the assay, *A. flavus* M3T8R4G3 and LAB inocula were prepared separately. *A. flavus* was cultured on PDA at 25 °C for 7 days. Spores were harvested using sterile 0.85% sodium chloride

solution containing 0.01% Tween 80, and the spore concentration was adjusted to 10^6 spores/mL using a hemocytometer. The selected LAB isolates were cultured on MRS agar under anaerobic conditions at 30 °C for 48 h. A single colony of each isolate was then inoculated into MRS broth and incubated at 30 °C for 48 h.

A 10 µL aliquot of each LAB culture was streaked as a straight line on one side of an MRS agar plate (90 × 15 mm) containing 20 mL of medium. A 10 µL aliquot of the fungal spore suspension was inoculated 2.5 cm from the LAB streak. The plates were incubated at 25 °C for 7 days in aerobic conditions to support the growth of fungal. *A. flavus* grown without LAB was used as the control. Fungal growth inhibition was calculated as follows:

$$\textbf{Growth inhibition (\%)} = [(C - T)/C] \times 100$$

Where *C* is the colony diameter of *A. flavus* grown without LAB (control), and *T* is the colony diameter of *A. flavus* grown in the presence of LAB.

2.3.2 Determination of culture pH

LAB isolates showing antagonistic activity against *A. flavus* were further evaluated for their ability to acidify MRS broth. Each isolate was activated on MRS plate agar under anaerobic conditions at 30 °C for 48 h. A single colony of each isolate was then inoculated into MRS broth and incubated at 30 °C for 48 h. After incubation, the pH of each culture was measured using a pH meter.

2.4 Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Mean and standard deviation were calculated using Microsoft Excel. Statistical analysis was performed using one-way analysis of variance (ANOVA) in SPSS version 28.0, followed by Tukey's post-hoc test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

3. Result and discussion

3.1 Silage fermentation quality

After 21 days of ensiling, the pineapple plant residues changed from bright green to yellowish-brown and became softer compared with the fresh material (Figure 1). These changes were clearly observed at the end of the ensiling period.

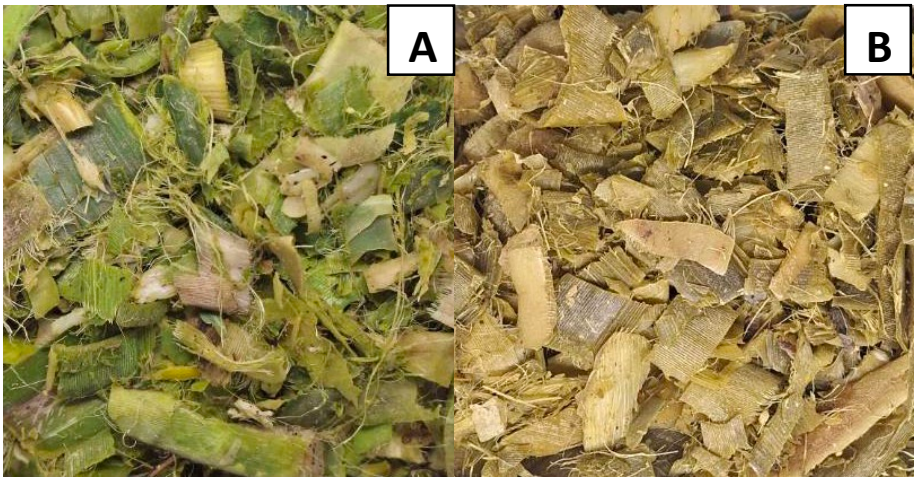

Figure 1. (A) fresh pineapple plant residue and (B) pineapple plant residue silage after 21 days ensiling

The pH changed during the 21-day ensiling period (Figure 2A). The initial pH was approximately 4.4 and increased during the early stage of ensiling. The pH ranged from 4.37 to 4.76 during the first 7 days and then decreased toward the end of the ensiling period. At day 21, the pH was approximately 4.5. Although a rapid decrease in pH is generally expected during silage fermentation, the pH remained below 5.0 throughout the ensiling period. The final pH was close to the reported pH range of 4.3–4.7 for grass silage [12].

The LAB population also changed during ensiling (Figure 2B). The initial population was 9.98 log CFU/g and decreased to 9.14 log CFU/g at day 1. It then increased to 9.89 log CFU/g at day 3, followed by a gradual decrease to 7.80 log CFU/g at day 21.

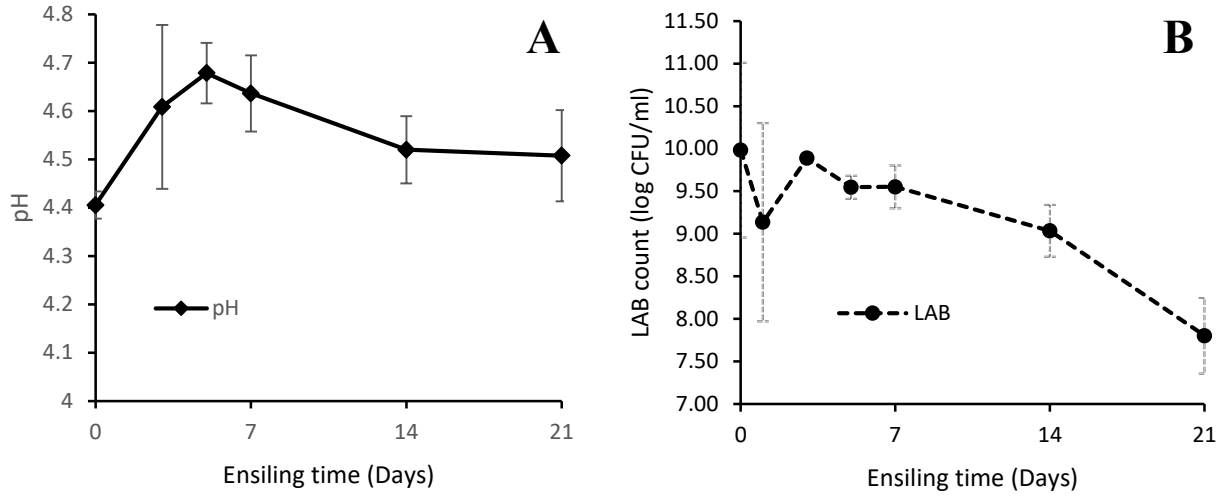

Figure 2. Changes in (A) pH and (B) LAB populations during ensiling of pineapple plant residues. Data are presented as mean ± SD (n = 3).

The changes in pH and LAB populations differed from the typical pattern of silage fermentation. Although LAB normally produce lactic acid and lower the pH, the pH increased during early ensiling, while the LAB population decreased at day 3 before increasing again at day 5. This suggests that rapid acidification did not occur during the early stage of ensiling. Lactic acid utilization by other microorganisms, competition for fermentable carbohydrates [2, 5, 16] and the buffering capacity of pineapple plant residues [15] may have contributed to this pattern.

Colonies showing clear zones on MRS agar supplemented with $CaCO_3$ were selected and purified. Based on Gram staining, cell morphology, spore formation, and catalase activity, 300 isolates were considered presumptive LAB and subjected to further screening.

3.2 Screening and selection of LAB isolates

The 300 presumptive LAB isolates were screened for acid-producing ability on MRS agar supplemented with $CaCO_3$ (Figure 3). Acid production was indicated by the formation of a clear zone around the colonies, and acid-producing ability was expressed as the ratio of clear-zone diameter to colony diameter. The ratios ranged from 1.22 ± 0.07 to 2.66 ± 0.15. Based on the selection criterion of ≥2.00, 196 isolates (65%) were selected, while 104 isolates (35%) had ratios below 2.00 (Figure 4).



Figure 3. Clear-zone formation by LAB isolates on MRS agar supplemented with $CaCO_3$ after 48 h of incubation at 30 °C.

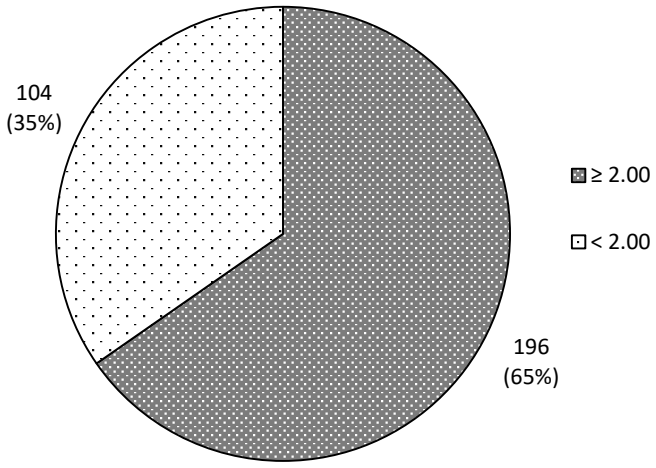

Figure 4. Distribution of presumptive LAB isolates based on acid-producing ability ratio.

The 196 isolates selected based on acid-producing ability were subsequently evaluated for antagonistic activity against *A. flavus* using the dual culture assay (Figure 5). All tested isolates inhibited fungal growth by more than 20%, although the level of inhibition varied among isolates. Of these, 90 isolates showing more than 35% growth inhibition were selected for further evaluation (Figure 6).

LAB with both antifungal activity and the ability to produce acidic conditions are desirable for use as silage starter cultures. The 90 isolates showing more than 35% inhibition against *A. flavus* were therefore further evaluated for acid production in liquid culture. After 48 h in MRS broth, the final culture pH ranged from 3.68 to 3.50, with a mean of 3.56 ± 0.03.

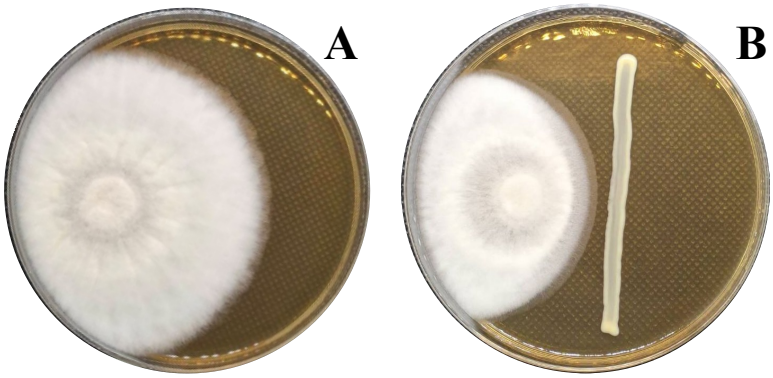

Figure 5. Antagonistic activity of LAB against *Aspergillus flavus* on MRS agar after incubation at 25 °C for 7 days: (A) control without LAB and (B) co-culture with a LAB isolate.

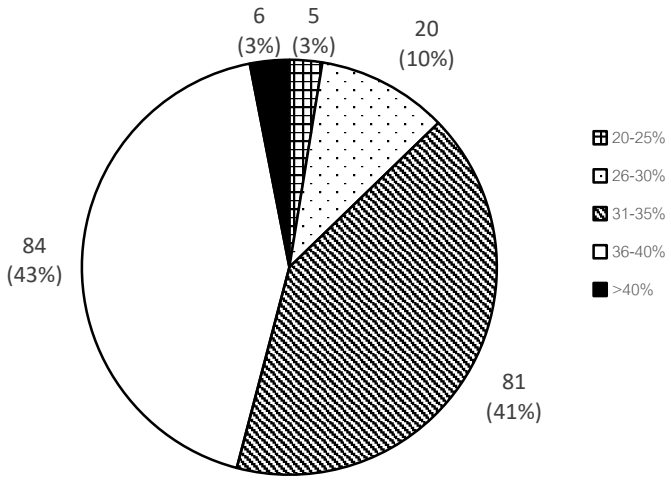

Figure 6. Distribution of LAB isolates based on growth inhibition against *A. flavus*.

The low final pH produced by the selected isolates is a desirable characteristic for silage fermentation, where effective LAB should rapidly establish acidic conditions that favor preservation. However, acid production alone may not be sufficient for selecting suitable starter cultures, particularly when fungal contamination is also a concern. The antifungal activity observed among the isolates therefore provides an additional functional property that may be useful for silage preservation. Previous studies have also reported inhibition of *A. flavu* by LAB, although antifungal activity varies considerably among strains [17]. identified only a small number of active isolates from a larger collection of LAB from cereal-based fermentation starters, indicating that antifungal activity is a strain-dependent characteristic. Thus, screening for both acid production and antifungal activity may be more useful than selection based on acid production alone when identifying LAB for further development as silage starter cultures.

4. CONCLUSIONS

Post-harvest pineapple plant silage was a source of indigenous LAB with acid-producing and antifungal properties. Selected isolates were able to lower the pH of MRS broth and inhibit *A. flavus*,

indicating their potential as starter cultures for pineapple plant silage. Further screening, identification, and characterization of the selected isolates are required to identify the most promising strains before evaluation under actual ensiling conditions.

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