Isolation and Characterization of Lactic Acid Bacteria Used for Ensiling Bamboo-shoot Shell

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ABSTRACT

Aims: To construct a stable and functional lactic acid bacteria community which can be used in the fermentation of bamboo-shoot shell to improve the tasty and flavor.

Methodology: Using naturally fermented bamboo-shoot shell as initial sample, a qualitative microorganism source had been prepared by the technique of continuous restricted subcultivation. Then some colonies producing acid were selected by plate isolation and streaking from above source. Morphology & phenotype experiments, catalase test, GC-MS and 16S rRNA analysis were employed to identify the isolates. Finally, the ensilage experiment of moist bamboo-shoot shell was carried out to assess the potency of the selected lactic acid bacteria.

Results: After a serial of subcultivation, a stable and functional bacteria community producing acid had been constructed in the 23rd culture of bamboo-shoot shell. The bacteria community consisted of Lactobacillus plantarum subsp. plantarum, Lactobacillus plantarum, Lactobacillus sp. T2R2C12 and Lactobacillus pentosus. Inoculated with the lactic acid strains, the culture pH of bamboo-shoot shell decreased sharply, meanwhile, the accumulation of lactic acid amounted to 4.01% at the end of ensiling.

Conclusion: Continuous restricted subcultivation is an effective technique to construct lactic acid bacterial community. We isolated several strains of lactic acid bacterial successfully from naturally fermented bamboo-shoot shell. The strains can participate in the fermentation of forage made of bamboo-shoot shell.

Keywords: bamboo-shoot shell; lactic acid bacteria; continuous restricted subcultivation; ensilage; forage.

1. INTRODUCTION

Moist bamboo-shoot shell, a kind of by-product from bamboo-shoot processing, is abundant in various organic components including cellulose (41.66%), hemicellulose (28.12%), lignin (20.34%) and other minor amount substances such as pectin, water-soluble carbohydrates and amino acids [1]. In the harvesting period of bamboo-shoot, huge number of moist shell was accumulated in a short time. It was estimated that moist bamboo-shoot shell exceeds million tons every year in China [2]. However, most of them could not be recycled, and was discarded in-situ. This treatment has caused serious pollution to the local environment. To date, bamboo-shoot shell has been developed for other new applications. For examples, the cellulose of bamboo-shoot shell can be used in textiles [3], dietary fibers [4], paper-pulps [5] and pharmaceuticals [6]. In fact, moist bamboo-shoot shell can also be converted into forage by ensiling [7]. In other words, ensiling can enhance the preservation of moist bamboo-shoot shell and prevent origin environment from polluting by bamboo shell spoilage.

Lactic acid bacteria (LAB) are considered to be the dominant microorganisms during silage fermentation. Dozens of species exist in the genus Lactobacillus [8]. In nature, these...
different species often compose a complex and synergetic community [9, 10]. It’s very difficult for researchers to identify all bacteria living in a natural community by any simple technique. Usually, researchers have to use a combination of different approaches to overcome the difficulty. Continuous restricted subcultivation is an efficient technique to construct a functional microbial community. Modern molecular technology such as denaturing gradient gel electrophoresis (DGGE), 16S rRNA analysis and other omics technology can assist researchers to investigate an interesting microbial community. Traditional techniques combined with modern molecular approaches had been successfully applied in numerous bacteria exploitation [10, 11, 12, 13].

Moist bamboo-shoot shell is a kind of organic resource. In this study, we attempted to isolated several LAB strains which could participate in the ensiling of bamboo-shoot shell. According to our purpose, naturally fermented bamboo-shoot shell should be considered as the best bacteria source. The whole technical route should also combine traditional and modern approaches.

2. MATERIALS AND METHODS

2.1 Preparation of Naturally Fermented Bamboo-shoot Shell

Moist bamboo-shoot shell was collected from Maosheng Food Company, Ningguo City, Anhui Province, P. R. China. The bamboo-shoot shell was chopped to about 2.0 cm and stored in screw-capped test tubes (50 mL), then the tubes were sealed tightly and cultivated at 30°C. After 9 days of fermentation, the tubes with pH<4.0 and specific acid flavor were chosen as naturally fermented bamboo-shoot shell.

2.2 Isolation and Purification of strains producing acid

Naturally fermented bamboo-shoot shell was transferred into de Man-Rogosa-Sharpe (MRS) broth at the ratio of 10% and cultivated for 3 days at 30°C. Then the MRS culture broth was transferred into fresh MRS and MRS-S (the carbon source was changed from glucose to sucrose) broth at the ratio of 5%, the inoculated broth were incubated for 3 days at 30°C. After continuous subculturing 30 times, the broth with a stable rate of pH decline and producing a large amount of acid were selected for the next plate isolation.

The selected broth was plated on MRS agar (added with 1.6% bromcresol-purple ethanol solution at the ratio of 0.2%, pH 7.2±0.2) after serial dilution. Inoculated MRS-S agar plates were overlaid with pure agar and incubated in an anaerobic jar for 48h at 30°C. Some colonies with yellow zone were picked up and purified by streaking on MRS agar [supplemented with 2.0% CaCO₃ (w/v), colony producing acid could form clear zone.]. The purified strains were maintained on MRS agar slants.

2.3 Identification of Isolated strains

Overnight-incubated cultures of isolated strains were Gram stained and examined microscopically for morphology. Catalase test was performed by adding few drops of 3% hydrogen peroxide to a microscope slide containing 24h old culture of each isolate.

After 72h cultivation, the MRS broth of isolated strains was measured by a combination of gas chromatography and mass spectrometry (SCION SQ GCMS, Bruker Daltonics Inc. USA) with a capillary column (DB-5MS, 60m×0.25mm, Agilent), and helium (64 kPa) used as the carrier gas. The column temperature was 50°C (2 min) → 100°C, 5°C min⁻¹ → 190°C (2 min), 15°C min⁻¹; injector temperature: 180°C; detector temperature: 230°C; rate of flow 30 mL min⁻¹; splitter ratio: 1/22; sample volume: 1µL; detector: 1.5 kV. The culture broth
samples were diluted for quantitative analysis. Data analysis: Firstly, a calibration curve could be created by finding the peak square of a series of concentrations of standard lactic acid. Subsequently the regression equation could be obtained. Finally the concentration of lactic acid in each broth could be calculated from the regression equation.

Genomic DNA from all isolates was extracted using Bacteria Genomic DNA Extraction Kit (TaKaRa Biotechnology, Dalian Co., Ltd, China) following the manufacturer's protocol. The amplification of 16S rRNA gene sequence was performed with universal primers of *E. coli* 16S rRNA gene sequence from 18-27 bp as forward (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1471-1492 bp as reverse primer (5'-TAC GGC TAC CTT GTT ACG ACT T-3') as described by Mahanteshl [11]. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation of 94°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 5 min. The PCR products of the 16S rDNA was purified sequenced in Sangon Biotechnology Co., Ltd, China. The similarity of sequence were analyzed in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) using the blastn database.

2.4 Assessment of Isolated LAB Community on Ensiling Bamboo-shoot Shell

Moist bamboo-shoot shell was chopped to about 2.0 cm and autoclaved. Then the chopped shell was inoculated with the mixture of isolated LAB suspension and ensiled in 50 mL tubes cultivated for 30 days at 30°C. The inoculants were applied as follows: The four isolated LAB were co-cultivated in MRS medium for 48h at 30°C, 1 mL MRS culture was centrifuged at 10,000 g for 1 min; after removing the supernatant the cells were suspended in 10 mL sterile distilled water, then mixed with 100 g chopped shell. Control was mixed with sterile distilled water at the same ratio.

After fermentation, three tubes of each treatment were sampled for chemical analysis including pH, lactic acid, crude protein, crude fiber and crude fat. The pH was measured by pH meter (METTLER TOLEDO FE20, China). Lactic acid levels were determined by the method described above. Crude protein, crude fat and crude fiber were analyzed according to the procedures described in reference [14].

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of LAB

3.1.1 Isolation of strains producing acid

After 23rd subcultivation, the pH of MRS broth could decrease to 3.18 within 72h and remain constant as shown in Fig. 1. As such, the 23rd broth was selected as bacteria source. Four colonies of acid-producing bacteria were picked up and purified from the MRS agar plate and named 1#, 2#, 3#, 4#.

3.1.2 Identification of LAB

3.1.2.1 The result of morphology experiment and catalase test

The isolates were Gram positive, catalase negative. Their cell morphology and colony morphology were described in Table 1.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colony morphology (48 h)</th>
<th>Cell morphology (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1#</td>
<td>Creamy white, protrusions, smooth and round, 0.5-2mm colony diameter</td>
<td>Straight or slightly recurved rod-shaped, Single, pairs or short chains</td>
</tr>
<tr>
<td>2#</td>
<td>Creamy white, protrusions, smooth and round, 0.5-2mm colony diameter</td>
<td>Straight or slightly recurved rod-shaped, Single, pairs or short chains</td>
</tr>
<tr>
<td>3#</td>
<td>White colonies, bumps, round, smooth and neat edge, moist, 1-2.5mm colony diameter</td>
<td>Short rod-shaped, single or pairs</td>
</tr>
<tr>
<td>4#</td>
<td>Slight bulge, round, creamy white, smooth, lustrous, 1-2mm colony diameter</td>
<td>Short rod-shaped, single or pairs</td>
</tr>
</tbody>
</table>

### 3.1.2.2 The measurement result of broth by GC-MS

The MRS broth of the isolates was measured by GC-MS. Under the constant conditions, the analytes in broth had the same retention time with the standard lactic acid (Fig.1). It suggested that all the four isolates could synthesize lactic acid. Based on the regression equation (Y = 6.9075X+0.2018, correlation coefficient r=0.9986), the lactic acid concentration of broth was: 1#- 4.52 mg/mL, 2#-4.76 mg/mL, 3#- 6.42 mg/mL and 4#-6.79 mg/mL.

![Gas chromatogram](image1.png)

**Fig.1.** The gas chromatogram of the isolates' broth after cultivated 72h in MRS-S medium (a, Isolate 1#; b, Isolate 2#; c, Isolate 3#; d, Isolate 4#; e, standard lactic acid)

### 3.1.2.3 Analysis result of 16S rRNA gene
As shown in Table 2, the four isolates have more than 99% similarities with *Lactobacillus plantarum subsp. plantarum*, *Lactobacillus plantarum*, *Lactobacillus sp* T2R2C12 and *Lactobacillus pentosus*, respectively.

According to the results from 3.1.2.1, 3.1.2.2 and 3.1.2.3, it could be considered that we had isolated different LAB from a single microbial source.

Table 2. BLAST analysis of the four lactic acid bacteria

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Closest relative</th>
<th>% Identity</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1#</td>
<td><em>Lactobacillus plantarum subsp.</em></td>
<td>99%</td>
<td>AB601168.1</td>
</tr>
<tr>
<td>2#</td>
<td><em>Lactobacillus plantarum</em></td>
<td>99%</td>
<td>CP006033.1</td>
</tr>
<tr>
<td>3#</td>
<td><em>Lactobacillus sp T2R2C12</em></td>
<td>99%</td>
<td>JX193619.1</td>
</tr>
<tr>
<td>4#</td>
<td><em>Lactobacillus pentosus</em></td>
<td>99%</td>
<td>FJ386571.1</td>
</tr>
</tbody>
</table>

It was well known that nearly all reactions of substance conversion usually can’t be completed by a single strain. Most conversion reactions must rely on two or more kinds of microbes [15]. In order to obtain a stable and functional microbial community for the ensiling of moist bamboo-shoot shell, the microorganism source employed in this study was not a traditional sample but fermented bamboo-shoot prepared by continuous restricted subcultivation. The isolates were all belonged to the genus *Lactobacillus*, which proved that the experiment method was very effective.

In course of the ensiling process, homofermentative lactic acid bacteria can lead silages to have low stability against aerobic deterioration [16], while heterofermentative lactic acid bacteria can enhance the stability [17]. It was reported that the inoculation with a mixed LAB inoculants (contained homofermentative lactic acid bacteria and heterofermentative ones) could enhance aerobic stability and, in general, reduce yeast and mould counts. In present study, both homofermentative and heterofermentative LAB were found in a community suggesting that the two kinds of organisms are probably a pair of close allies in nature.

### 3.2 Effect of LAB Community on Ensiling Moist Bamboo-shoot shell

After 30 days of fermentation, the culture pH decreased significantly from 6.20 to 3.23, meanwhile its concentration of lactic acid reached 4.01%. However, there were little changes in crude protein, crude fat and crude fiber. And the content of water-soluble carbohydrate (WSC) reduced slightly due to the consumption of LAB, as shown in Fig. 2.

In addition, a trace amount of acetic acid and ethanol was also measured in the culture (data not shown). All the results indicated that the selected LAB had the potency on ensiling bamboo-shoot shell.
Based on the medium of bamboo-shoot shell, the isolated LAB strains could synthesize lactic acid even acetic acid and ethanol which often benefit the preservation, aerobic stability and flavor of silage. At present China, moist bamboo-shoot shell is usually regarded as a kind of waste and treated irregularly. Present study demonstrated that the waste could be developed into a new kind of forage. Nevertheless, LAB can’t degrade lignocellulose. The product of lignocellulose degradation is the substrate of LAB, LAB can enhance the preservation and improve the nutrition value of bamboo-shoot shell. During the following work, we’ll investigate the effect of inoculants mixed with LAB and Aspergillus niger, a producer of cellulose, on bamboo-shoot shell.

4. CONCLUSION
A stable microorganism community producing acid was constructed and isolated successfully from the naturally fermented bamboo-shoot shell. The results of identification suggested that the community consisted of lactobacilli. Ensilage of bamboo-shoot shell indicated that the LAB community could participate in the ensiling of forage.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

REFERENCES


APPENDIX