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

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Authors' contributions

This work was carried out in collaboration between all authors. Author WZJ designed the study. Author ZJG conducted statistical analysis and wrote the first draft of the manuscript. Author LQL performed the experiments. All authors read and approved the final manuscript.

Original research Article

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ABSTRACT

Aims: To construct a stable and functional lactic acid bacteria community that can be used in the ensilage of bamboo-shoot shell to improve the taste 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 sub cultivation. 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 sub cultivation, a stable and functional bacteria community producing acid had been constructed in the 23rd culture of bamboo-shoot shell. The bacteria community consisted of *Lacto bacillus plantarum* subsp. *plantarum*, *Lactobacillus plantarum*, *Lactobacillus* spp. *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 sub cultivation is an effective technique to construct lactic acid bacterial community. Four strains of lactic acid bacterial were isolated successfully from naturally fermented bamboo-shoot shell. The strains can participate in the fermentation of forage made of bamboo-shoot shell.

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Keywords: Bamboo-shoot shell; lactic acid bacteria; continuous restricted sub cultivation; 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, nearly all bamboo-shoot shell could not be recycled, and was discarded in-situ. This way 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]. Ensiling can enhance the preservation of moist bamboo-shoot shell and prevent origin environment from polluting by bamboo shell spoilage.

Lactic acid bacteria (LAB) were considered to be the dominant microorganisms during silage fermentation. There are dozens of species in the genus *Lactobacillus* [8]. In nature, these different species often compose a complex and synergetic community [9, 10]. It was very difficult to identify all bacteria living in a natural community by any simple technique. Usually, combination methods of different approaches were used to overcome the problem. Continuous restricted subcultivation was 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 could be used to investigate an interesting microbial community. Traditional techniques combined with modern molecular approaches had been successfully applied in numerous bacteria exploitation [10-13].

In this study, moist bamboo-shoot shell was a kind of organic substance resource. It was attempted to isolated several LAB strains which could participate in the ensiling of bamboo-shoot shell. According to this 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 2.0cm and stored in screw-capped test tubes (50mL), 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 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 sub cultivation for 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 [10].

The selected broth was plated on MRS-S 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% $CaCO_3$ (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 [14].

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 (64kPa) used as the carrier gas. The column temperature was 50°C (2min) → 100°C, 5°Cmin^{-1} → 190°C (2min), 15°Cmin^{-1}; injector temperature: 180°C; detector temperature: 230°C; rate of flow 30mL min^{1}; splitter ratio:1/22; sample volume:1μL; detector:1.5kV [10,15]. The culture broth samples were diluted for quantitative analysis. Data analysis: Firstly, a calibration curve would be created by finding the peaks 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 individually using Bacteria Genomic DNA Extraction Kit (TaKaRa Biotechnology, Dalian Co., Ltd, China) following the manufacturer's protocol. The amplification of 16SrRNA gene sequence was performed with universal primers of *E. coli* 16SrRNA gene sequence from 18-37bp as forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1471-1492bp 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 5min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30sec and extension at 72°C for 5min. The PCR products of the 16SrDNA were purified and sequenced in Sangon Biotechnology Co., Ltd, China. The similarities of sequence were analyzed in Gen Bank (http://www.ncbi.nlm.nih.gov/BLAST/) using the blastn database.

2.4 Assessment of Isolated LAB on Ensiling Bamboo-shoot Shell

Moist bamboo-shoot shell was chopped to about 2.0cm and autoclaved. Then the chopped shell was inoculated with the mixture of isolated LAB suspension and ensiled in 50mL tubes cultivated for 30 days at 30°C. The inoculation was conducted as follows: The isolated LAB were co-cultivated in MRS broth for 48h at 30°C, 1mL of MRS culture broth was centrifuged at 10,000g for 1min; after removing the supernatant the cells were suspended in 10mL 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 [16]. These data were analyzed using SPSS (version 19.0th) by paired-samples T test.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of LAB

3.1.1 Isolation of strains producing acid

The result of pH tendency during continuous restricted sub cultivation was shown in Fig. 1. From the 23rd sub cultivation, the pH of MRS-S culture broth could decrease to 3.18 within 72h and remain constant. As such, the 23rd broth was selected as bacteria source. After plate isolation and purification, four strains were obtained and named 1#, 2#, 3# and 4#.

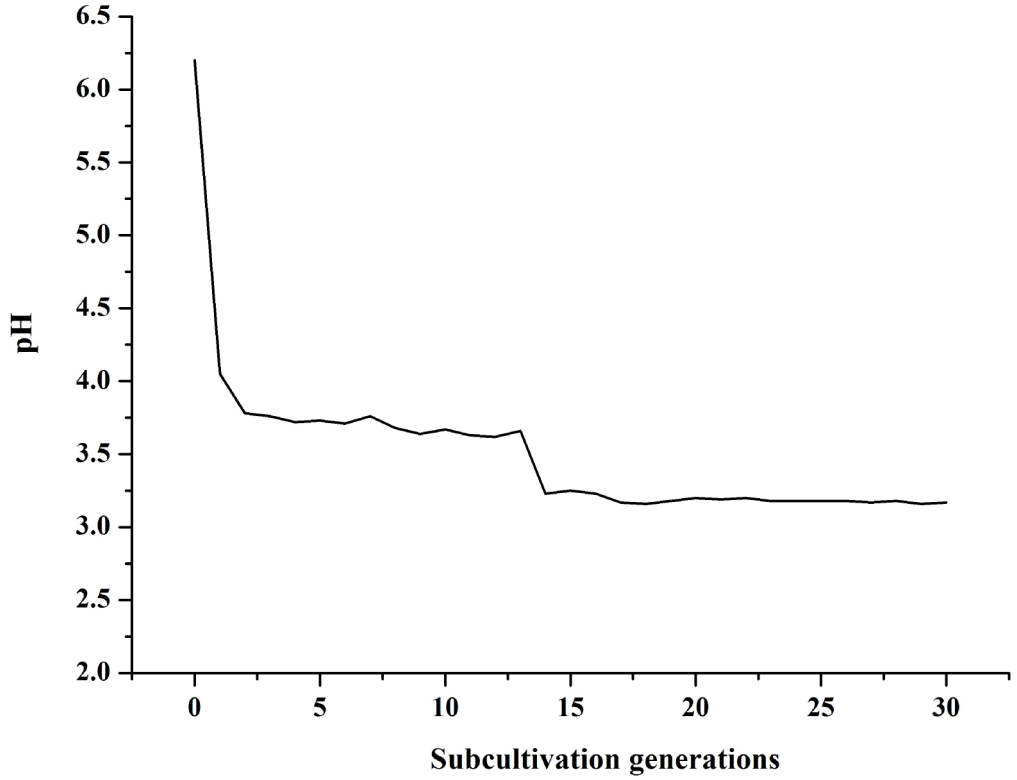

Fig. 1. The chart of pH tendency during continuous restricted sub cultivation

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 1. Cell and colony morphology of the isolates

Isolate	Colony morphology (48h)	Cell morphology (48h)
1#	Creamy white, protrusions, smooth and round, 0.5-2mm colony diameter	Straight or slightly recurved rod-shaped, Single, pairs or short chains
2#	Creamy white, protrusions, smooth and round, 0.5-2mm colony diameter	Straight or slightly recurved rod-shaped, Single, pairs or short chains
3#	White colonies, bumps, round, smooth and neat edge, moist, 1-2.5mm colony diameter	Short rod-shaped, single or pairs
4#	Slight bulge, round, creamy white, smooth, lustrous, 1-2mm colony diameter	Short rod-shaped, single or pairs

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. 2. 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 calculated: 1#-4.52mg/mL, 2#-4.76mg/mL ,3#-6.42mg/mL and 4#- 6.79mg/mL.

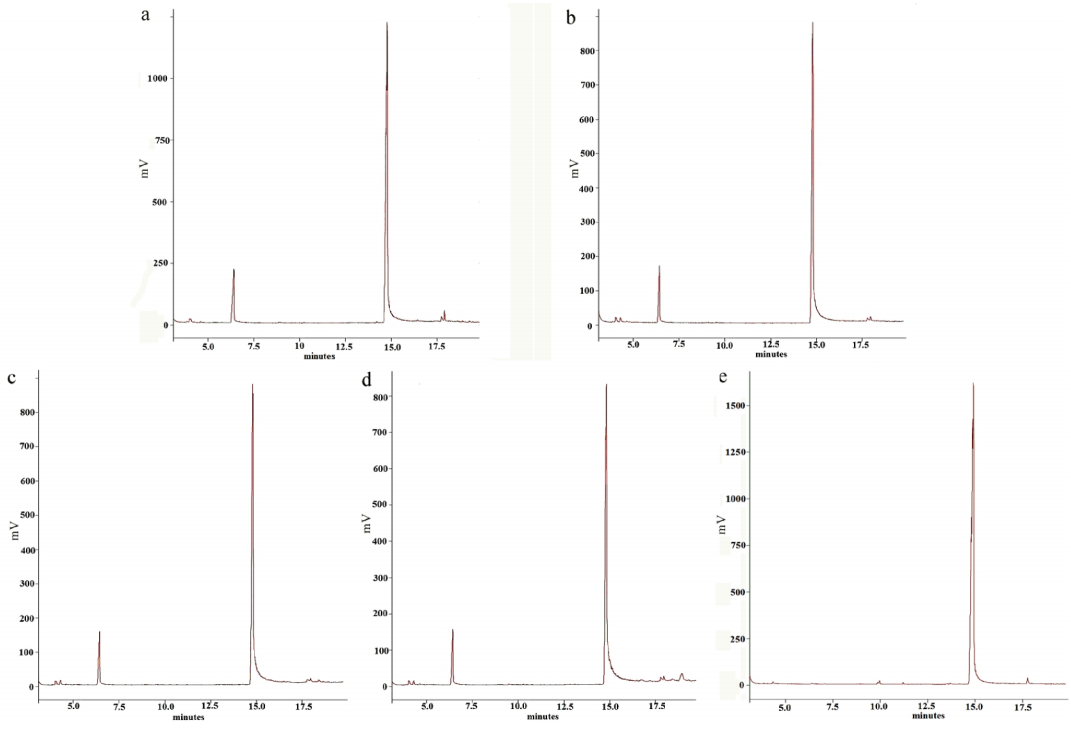

Fig. 2. The gas chromatogram of the isolates' broth after cultivated 72h in MRS medium (a, Isolate 1#; b, Isolate 2#; c, Isolate 3#; d, Isolate 4#; e, standard lactic acid)

3.1.2.3 Analysis result of 16SrRNA gene

Four isolates were more than 99% similarities with *Lactobacillus plantarum* subsp. *plantarum*, *Lactobacillus plantarum*, *Lactobacillus* spp. *T2R2C12* and *Lactobacillus pentosus*, respectively Table 2.

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

Table 2. BLAST analysis of the four lactic acid bacteria

Isolate	Closest relative	% Identity	Accession number
1#	*Lactobacillus plantarum* subsp. *plantarum*	99%	AB601168.1
2#	*Lactobacillus plantarum*	99%	CP006033.1
3#	*Lactobacillus* spp. *T2R2C12*	99%	JX193619.1
4#	*Lactobacillus pentosus*	99%	FJ386571.1

It was well known that symbiotic interaction of various bacteria could enhance the ability of metabolism in nature. Most conversion reactions must rely on two or more kinds of microbes [17]. In order to obtain a stable and functional microbial community for the ensiling of moist bamboo-shoot shell, the microorganism source was not a traditional sample but fermented bamboo-shoot prepared by continuous restricted sub cultivation in this study. The isolates were all belonged to the genus *Lactobacillus*, which proved that the experiment method was very effective.

In course of the ensiling, homo fermentative lactic acid bacteria can lead silages to have low stability against aerobic deterioration [18], while hetero fermentative lactic acid bacteria can enhance the stability [19]. It was reported that the inoculation with a mixed LAB inoculants (contained homo fermentative lactic acid bacteria and hetero fermentative ones) could enhance aerobic stability and, in general, reduce yeast and mould counts. In present study, both homo fermentative and hetero fermentative 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 Isolated LAB on Ensiling Moist Bamboo-shoot shell

After 30 days of fermentation, the culture pH was decreased significantly from 6.20 to 3.23, meanwhile its concentration of lactic acid was up to 4.01%. However, there were no significant differences in crude protein, crude fat and crude fiber. The content of water-soluble carbohydrate (WSC) reduced slightly due to the consumption of LAB, as shown in Fig. 3.

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 isolated LAB had the potency on ensiling bamboo-shoot shell.

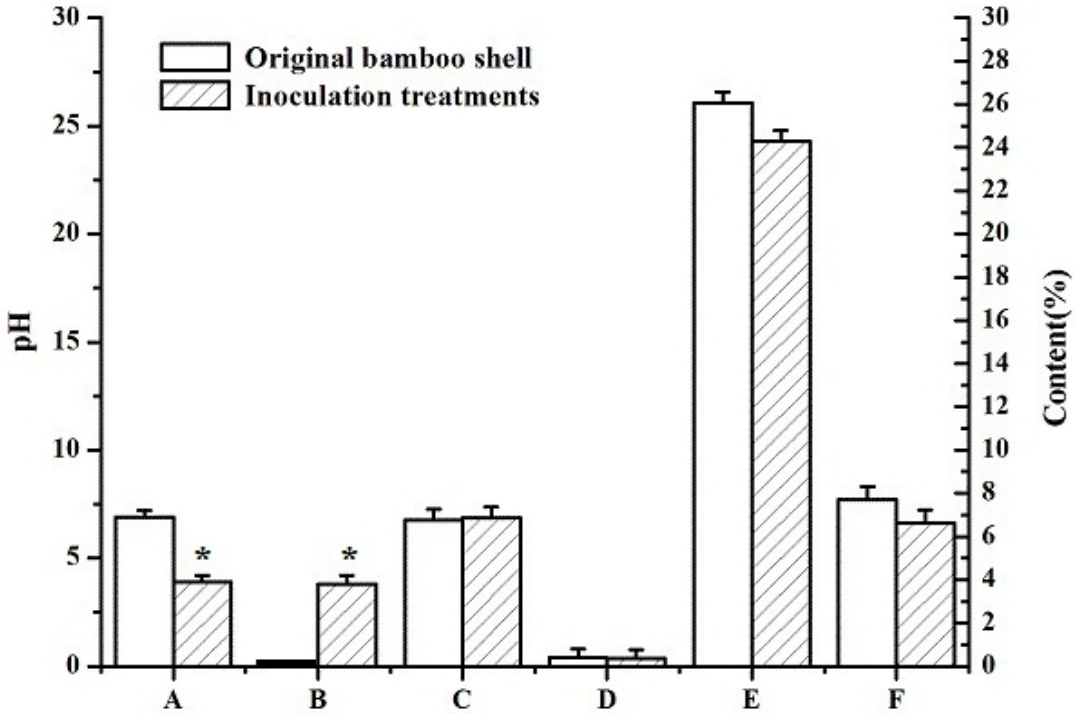

Fig. 3. Changes of pH and main organic components during ensiling of bamboo-shoot shell inoculated with isolated LAB (A, pH; B, lactic acid; C, crude protein; D, crude fat; E, crude fiber; F, water-soluble carbohydrate) **P< 0.05*

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. In China, moist bamboo-shoot shell was 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 was the substrate of LAB, LAB can enhance the preservation and improve the nutrition value of bamboo-shoot shell. During the following work, the effect of inoculants mixed with LAB and *Aspergillus niger*, a producer of cellulase, on bamboo-shoot shell will be investigated.

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 isolated LAB community could participate in the ensiling of forage.

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CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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