

Development of an Enrichment Culture Growing at Low Temperature used for Ensiling Rice Straw

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To speed up the conversion of rice straw into feeds in a low-temperature region, a start culture used for ensiling rice straw at low temperature was selected by continuous enrichment cultivation. During the selection, the microbial source for enrichment was rice straw and soil from two places in Northeast China. Lab-scale rice straw fermentation at 10°C verified, compared with the commercial inoculant, that the selected start culture lowered the pH of the fermented rice straw more rapidly and produced more lactic acid. The results from denatured gradient gel electrophoresis showed that the selected start culture could colonize into the rice straw fermentation system. To analyze the composition of the culture, a 16S rRNA gene clone library was constructed. Sequencing results showed that the culture mainly consisted of two bacterial species. One (A) belonged to *Lactobacillus* and another (B) belonged to *Leuconostoc*. To make clear the roles of composition microbes in the fermented system, quantitative PCR was used. For species A, the DNA mass increased continuously until sixteen days of the fermentation, which occupied 65%. For species B, the DNA mass amounted to 5.5% at six days of the fermentation, which was the maximum relative value during the fermentation. To the authors' best knowledge, this is the first report on ensiling rice straw with a selected starter at low temperature and investigation of the fermented characteristics.

Keywords: Rice straw, ensile, low temperature, enrichment cultivation, quantitative PCR

Rice straw is one of the abundant lignocellulosic waste materials in the world. According to the estimation by

FAO in 2004, annual production of rice is about 600 million tons. Moreover, every kilogram of grain harvested is accompanied by production of 1–1.5 kg of the straw [9]. Based on these data, 75–110 million tons of rice straw were produced in China in 2005 [3].

In Northeast China, one of the main foodstuff-producing fields, the amount of rice straw accounted for twenty-seven percent of the whole country [3]. Drought and low temperature in autumn, winter, and spring become the limited factors to straw amendment. Although more attentions to convert this residue into energy are being paid, this work is still under development [12]. A considerable amount of rice straw is wasted. Field burning becomes the major practice for removing rice straw, but it increases the air pollution and consequently affects public health [15].

On the other hand, the feeds for ruminants are insufficient during the winter season. Rice straw directly served as feeds for a long time in this region. However, the lignocellulosic composition of rice straw after harvesting seriously affected the palatability of feeds for the ruminants [14]. Silage fermentation has proved an effective way to improve the feed palatability [25, 28]. Successful ensilage on rice straw can be achieved by inoculation [6]. Therefore, ensiling rice straw in this region will be a feasible way to transform residues into value-added products in this region. However, low temperature will become a key limiting factor for this transformation. In this region, the average temperature in autumn is approximately 6.3°C [21]. In general, the temperature for ensiling is from 20–40°C [13]. There are few reports on silage fermentation at lower temperature, especially on ensiling rice straw with the inoculation at lower temperature.

In this research, an enrichment culture growing at low temperature was selected by continuous enrichment cultivation due to a rapid decline in pH. Furthermore,

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characteristics of the growth, inoculating effects on rice straw compared with the commercial inoculant, and composition of the culture were investigated.

MATERIALS AND METHODS

Cultivation

The modified MRS (R-MRS) broth was used as cultivation substrates. Ten g of rice straw was added into one liter of MRS [2] broth. After sterilization, the broth was divided into 15-ml screw-capped test tubes. Approximately 0.5 g of rice straw and/or soil from two sampling places in Northeast China was inoculated into the test tube and cultivated at 5°C. At the same time, two enrichment cultures in our laboratory, A12 [24], SFC-2 [6], and three commercial inoculants (CI, Sil-All 4×4, U.S.A.; WeiChuWang, China; YiShengKang, China) used for general ensiling were cultivated as described above. After 10 d cultivation, 15 µl of culture was re-inoculated into a new tube with the R-MRS broth. For the following repetitive cultivation, the time interval was four days.

Ensiling

The rice straw was chopped up to approximately 1 cm for fermentation. Glucose (Wako, Japan) was used to adjust the water-soluble carbohydrate of rice straw to 7%DM [27]. Deionized water was sprayed on the rice straw and the final moisture was about 70% of the mixtures. The culture in this study or the commercial inoculant CI (Sil-All 4×4) was inoculated with the ratio of 1×10^6 CFU/g rice straw. The treatment without inoculation was used as the control for the inoculation. Finally, the mixtures were ensiled in 100-ml test-tube silos, and were allowed to ferment under anaerobic condition at 10°C.

Growth Analyses and Microbial Enumeration

The optical density (OD) of cultures after 4-day cultivation was measured at 540 nm in glass cuvettes with a 10 mm light path (Ultrospec 1100 pro, Biochrom LTD, U.K.). The lactic acid bacteria (LAB) enumeration was performed using serial 10-fold dilutions on the R-MRS agar. The plates were incubated for four days at 20°C.

Chemical Analysis

The pH was determined using the compact pH meter (model B-212; Horiba, Kyoto). For volatile fermented products, 1.5 g of fermented rice straw was dipped into a 10-ml test-tube containing 4.5 ml of sterile water for 30 min. After centrifugation ($5,800 \times g$) and filtration (0.22 µm), the fluids were analyzed by gas chromatography mass spectrometry (GC/MS) (Model GCMS-QP2010, SHIMADZU, Japan): 1 µl juice was injected into the capillary column (CP-Chirasil-Dex CB, 25 m×0.25 mm); split ratio was 20:1; injector temperature was 190°C; ion source temperature was 200°C; interface temperature was 200°C; detector voltage was 0.7 kV, carrying gas was helium; the column head pressure was 75 kPa; and the total flow rate was 34.1 ml/min. Temperature program: initial 60°C (hold 1 min), rate of 7°C/min to 100°C (hold 1 min), rate of 18°C/min to 195°C (hold 2 min). The data were analyzed by the SHIMADZU GCMS solution V2.4 data processing system. Each sample contained three replicates.

Data were subjected to ANOVA using the general linear model procedure of the Statistic Analysis System (Version 6.12, SAS, Inst. Inc., Cary, NC, U.S.A.).

Denaturing Gradient Gel Electrophoresis (DGGE) Analyses

The genomic DNAs were extracted by the benzyl chloride method [29]. The extracted DNA was used as the template for PCR amplification. The primers and the programs used for amplifying the V3 region of 16S rDNA were the same as described previously [27].

DGGE analysis was performed according to the reference [26]. The excised bands were re-amplified with the primers 357F (5'-CCTACGGGAGGCAGCAG-3') and 517R (5'-ATTACCGCGGCT-GCTGG-3') [8]. The amplified fragments were purified using a QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed with a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), and the products were analyzed with the ABI 3130xl genetic sequencer (Applied Biosystems).

Cloning

A bacterial 16S rRNA gene clone library was constructed according to a previous description [24]. A total of 200 white colonies was randomly picked and screened by DGGE profile as described above. The clones that produced a single band with different melting positions were selected for sequence analysis. The insert DNA fragments were sequenced using the primers T7, 515F, and Sp6 [18].

Phylogenetic Analysis

Sequence similarities were performed in GenBank using the Blastn database. The sequence information was then imported into the Clustal X software program for assembly and alignment [23]. The phylogenetic tree was constructed by the neighbor-joining method [19].

The sequences generated in this study were deposited with GenBank under the accession numbers EF590122-EF590135.

Quantitative PCR and Specific PCR Analyses

According to the results of the clone library, two isolates, which have the same sequences as the clone library (A-Clone 1 and B-Clone 2), were obtained. The DNA of each isolate was used as a template for the standard curve. The DNA extracted from the fermented rice straw was used as sampling templates. The concentration of total DNA as the template was determined using the Fluorescent DNA Quantification Kit. The quantifying process followed the instructions by the manufacturer (Bio-Rad, U.S.A.). Quantitative PCR was carried out using a LightCycler system (Roche Diagnostics) with Lightcycler-FastStart DNA Master SYBR green I (Roche Diagnostics). Each reaction mixture contained LightCycler-FastStart DNA Master SYBR Green I, 2 µl; 25 mM MgCl₂, 1.2 µl (for A) or 1.6 µl (for B); forward and reverse specific primers (10 pmol µl⁻¹), 1 µl each; and PCR-grade distilled water to give 18 µl. Finally, 2 µl of DNA solution was added. Each sample contained two replicates.

Specific primers were designed with the aid of PRIMROSE software [1] based on the sequence database of ribosomal database project II (RDP II). The designed primers were checked using the Probe_Match tool of RDP II. The specific primer pair for A was A-278F (5'-GGTAAAGGCTCACCAAGACC-3', *Escherichia coli*

positions 259 to 278) and A-467R (5'-TACCGTCACTACCTGAT-CAG-3', *E. coli* positions 467 to 486). For B, these were B-456F (5'-TGGGAAGAACAGCTAGAGTAG-3', *E. coli* positions 436 to 456) and B-612R (5'-TCCAATGCCTTTCCGGAGTT-3', *E. coli* positions 612 to 631). The PCR program was started with an initial denaturation at 95°C for 10 min. Subsequently, the target site was amplified in 40 cycles. Each cycle consisted of denaturation for 5 s at 95°C, annealing for 6 s at 65°C, and extension for 20 s at 72°C. Fluorescence was detected at the end of the extension reaction. The specificity of the amplified PCR product was assessed using a melting curve analysis [10].

RESULTS

Selection of the Enrichment Culture

The soil and rice straw obtained in two regions of Northeast China were used as the microorganisms sources. At the same time, two stable lactic acid bacteria (LAB) communities and three commercial inoculants were also cultivated. At the end of the first cultivation, most of the cultures could grow at 5°C. However, from the second cultivation, as repetitive cultivation progressed, most of them could not grow again. After 16 times of cultivation, one culture was selected for its higher OD and lower pH. According to the PCR-DGGE results (Fig. 1), the DGGE patterns of the culture did not change after the 16th repetitive cultivation. It was named SFL.

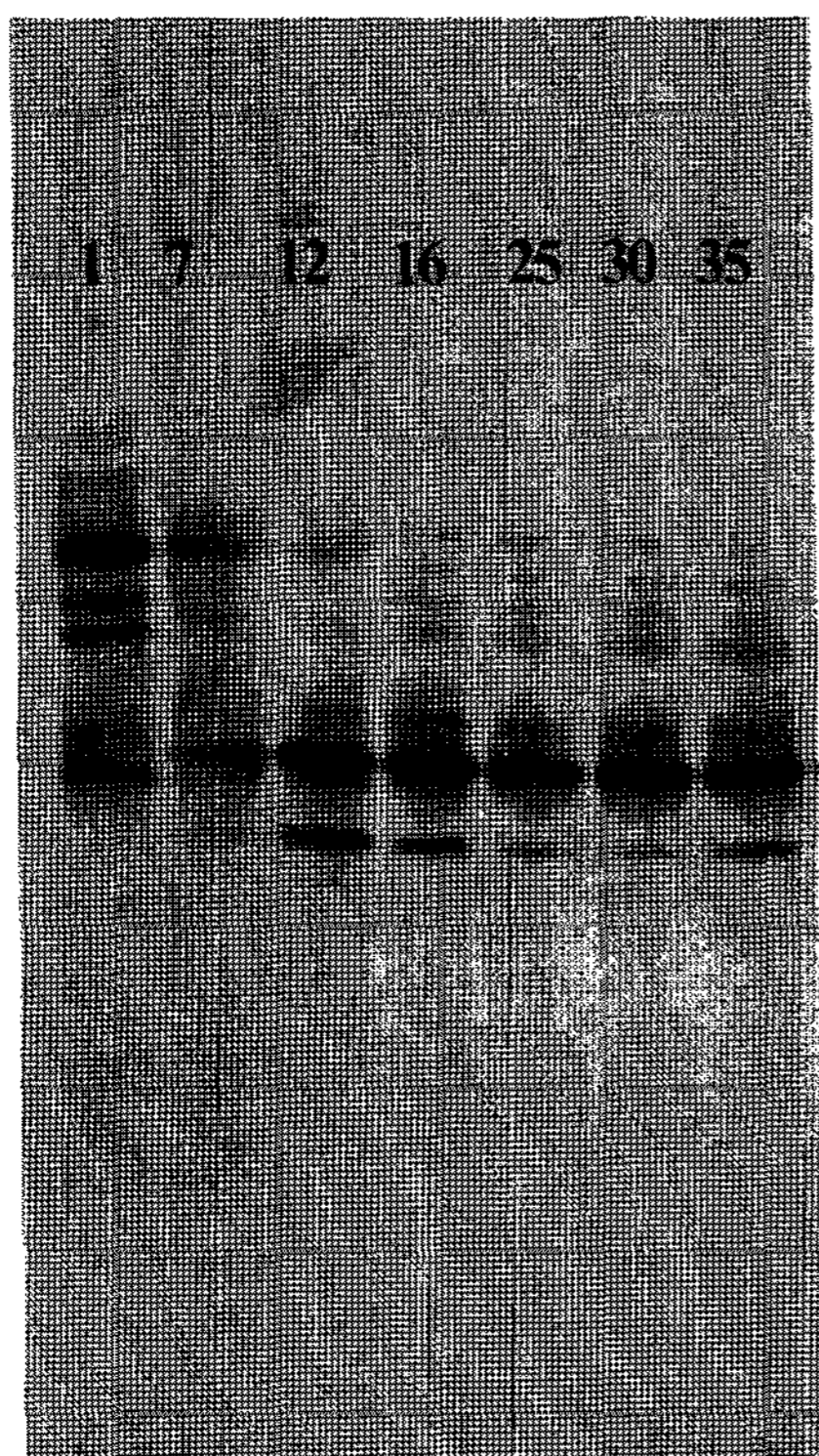


Fig. 1. DGGE profiles during the selection. Numbers represent times of repetitive cultivation.

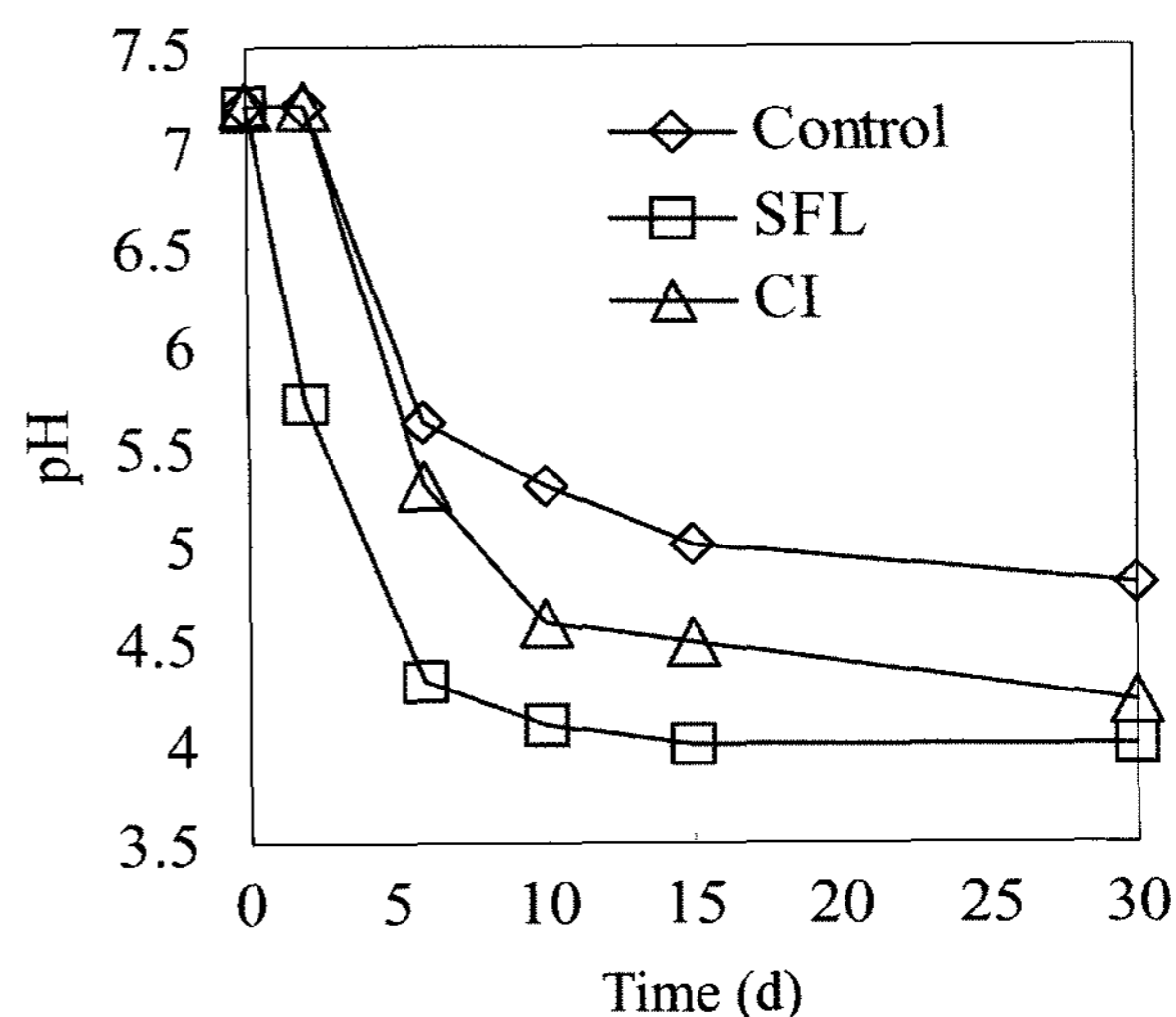


Fig. 2. Changes of pHs during the fermentation at 10°C. Control, no addition; SFL, the culture in this research; CI, the commercial inoculant.

Inoculating Effects at Low Temperature

To test the inoculating effects of the SFL on the rice straw at low temperature, the SFL and the commercial inoculant (CI) were inoculated into rice straw, respectively. The pHs, CFUs of LAB, and volatile fermented products were checked. The colonization of the SFL was checked using DGGE.

Changes of the pHs during the Fermentation

At 10°C, after 6 d of fermentation, pHs with inoculants were lower than the control (Fig. 2). The addition of the inoculants could distinctly speed up the decline in pHs of

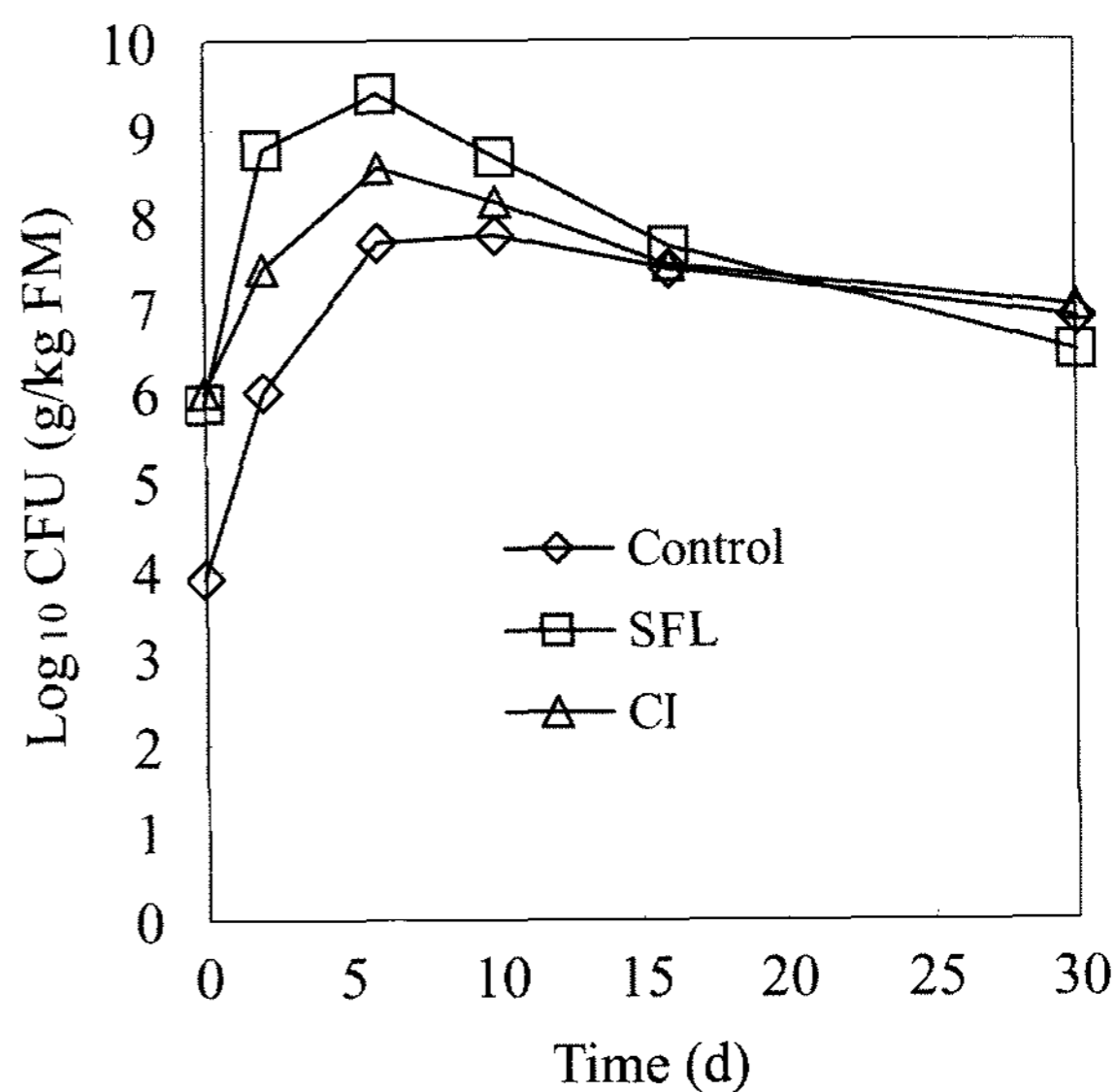


Fig. 3. Changes in CFUs of LAB at 10°C. CFU, colony forming unit; control, no addition; SFL, the culture in this research; CI, the commercial inoculant. FM, fresh matter.

Table 1. Volatile fermented products at 30 d fermentation of rice straw.

Temperature (°C)	Treatment	Ethanol	Acetic acid	Lactic acid	Glycerol
		g/kg FM*			
10	Control	0.22 ^c	1.14 ^b	0.03 ^b	0.31 ^a
	CI	1.59 ^a	0.72 ^c	2.03 ^b	0.39 ^a
	SFL	1.03 ^b	1.51 ^a	8.06 ^a	0.12 ^b

*FM - fresh matter; Control, no addition; CI, commercial inoculant; SFL, the culture in this research. Superscripts with different letters in the same column indicate significant difference ($P < 0.05$).

the rice straw. Furthermore, at 6 d of fermentation, the pH of the rice straw with SFL decreased to below 4.5. At 10 d of fermentation, the pH decreased to about 4.0. Compared with the commercial inoculant, the SFL could lower the pH more rapidly.

Microbiological Enumeration

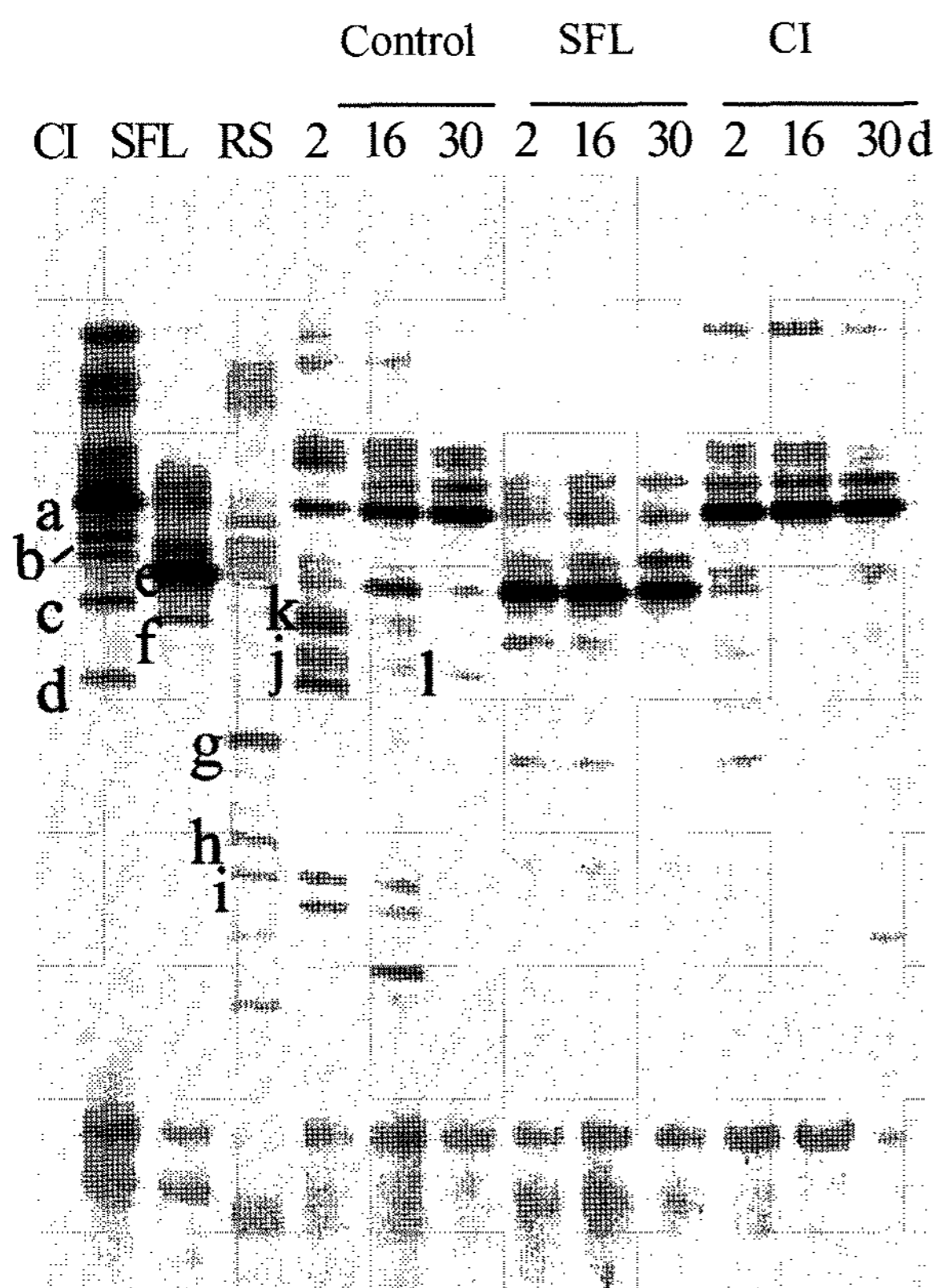
According to Fig. 3, the CFU of LAB of all treatments increased rapidly during the first 6 d of fermentation at 10°C. Then, the values began to decrease. Furthermore, the

LAB in the treatment with the SFL increased faster than the commercial inoculant at the beginning of the fermentation. At the end of the fermentation, it had a lower ($P < 0.05$) CFU than those in the commercial inoculant and the control.

Analyses of Volatile Fermented Products

Volatile fermented products were measured using GC/MS. The addition of the inoculants could significantly increase ($P < 0.05$) the ethanol concentration (Table 1). For glycerol, the inoculation did not significantly ($P < 0.05$) affect the concentrations in the fermented rice straw.

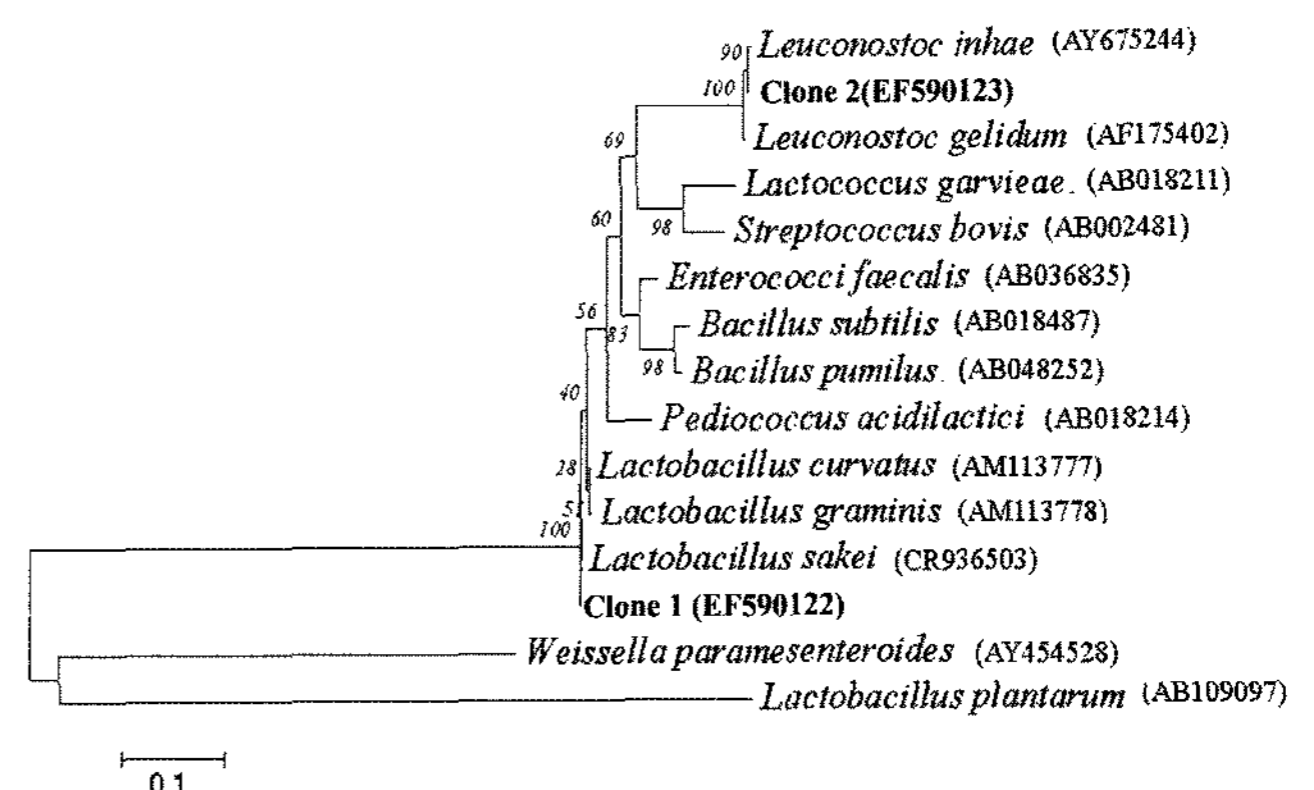
For lactic acid and acetic acid, the concentrations respectively amounted to 8.1 and 1.5 g/kg FM in the treatment with the SFL. The concentrations of these two matters with the SFL were significant higher ($P < 0.05$) than those with the CI.

**Fig. 4.** DGGE profiles of the samples at 10°C.

CI, commercial inoculant; SFL, the community in this research; RS, rice straw; control, no addition. a, *Lactobacillus plantarum* (100%); b, *Enterococcus faecium* (100%); c, *L. salivarilus* (100%); d, *Pediococcus acidilactici* (100%); e, *L. sakei* (100%); f, *Leuconostoc inhae* (100%); g, Uncultured bacterium (98%); h, Uncultured bacterium (97%); i, Uncultured bacterium (98%); j, *Pseudomonas lutea* (98%); k, Uncultured proteobacterium (100%); l, Uncultured *Pseudomonas* (99%).

Identification of the Colonization of the SFL during the Rice Straw Fermentation

To identify the colonization of the SFL during the rice straw fermentation, microbial compositions of the fermented rice straw at 6 d, 16 d, and 30 d were analyzed using PCR-DGGE (Fig. 4). At 10°C, throughout the fermentation, more microbial species were detected in the control. At 6 d of fermentation, fewer microbial species in the treatment with

**Fig. 5.** Phylogenetic tree derived from partial 16S rDNA sequence. The tree was constructed by the neighbor-joining method. Two hundred white colonies were detected: 158 colonies belonged to Clone 1, and 42 colonies belonged to Clone 2.

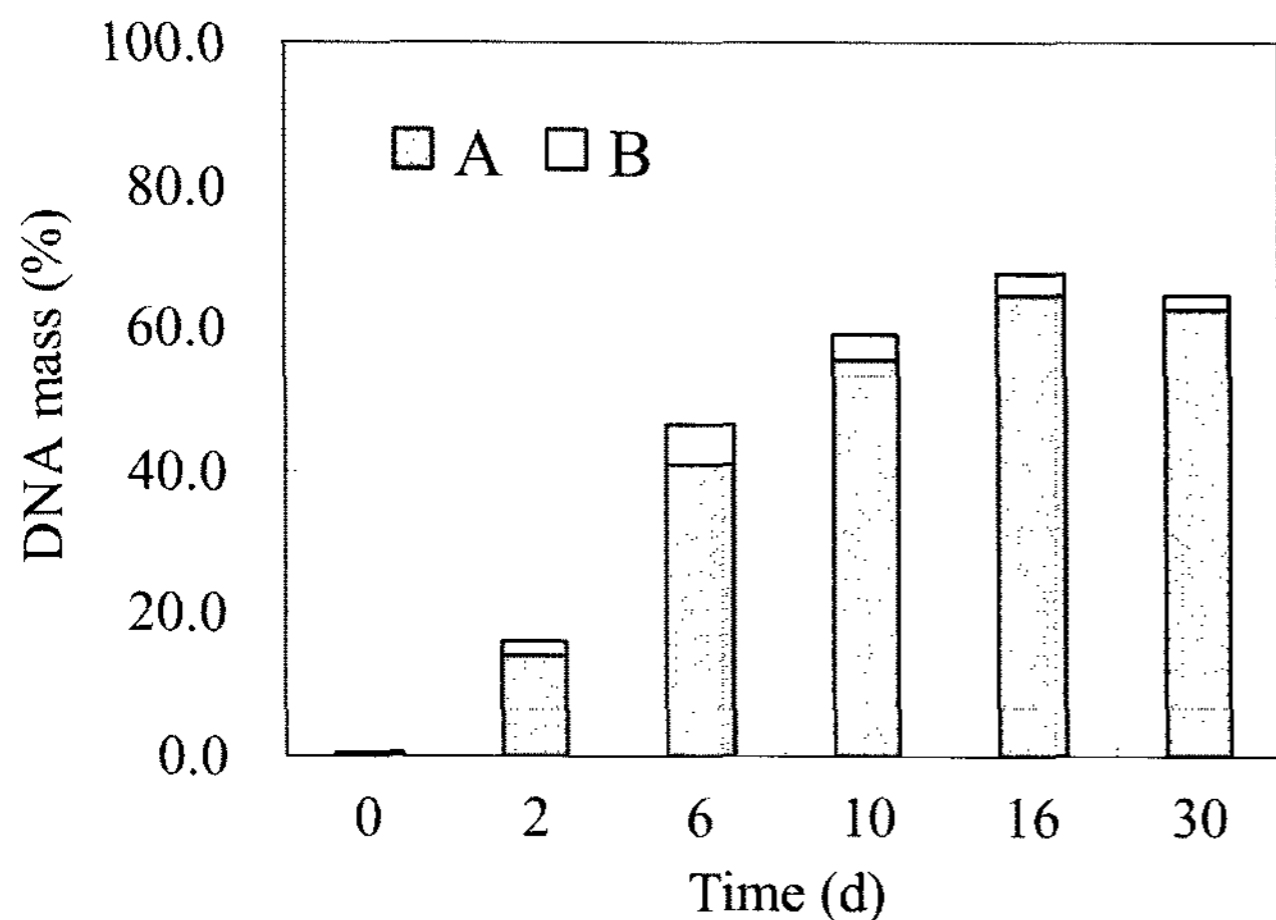


Fig. 6. Relative abundance of the composition microorganisms of the SFL during ensiling the rice straw.

A, *Lactobacillus sakei* (99%); B, *Leuconostoc inhae* (99%). Values are means of two replicates. DNA mass of A and B in the control was under the detection limit.

the SFL compared with the CI indicated that the fermentation occurred earlier. These results were in accordance with changes in pHs and the CFUs.

Composition of the SFL According to the Clone Library

According to the results of ensiling the rice straw, compared with the CI, the SFL lowered the pH of the rice straw more rapidly and produced more lactic acid at the low temperature. The compositions of the SFL were further investigated. Wang *et al.* [24] have reported that construction of a clone library is a preferable method to analyze the composition of the community. To analyze the composition of the SFL, a 16S rRNA gene clone library was constructed. The results of phylogenetic analyses are shown in Fig. 5. Sequencing results showed that the SFL mainly consisted of two bacterial species. One (A) belonged to the *Lactobacillus*, and another (B) belonged to the *Leuconostoc*.

Dynamics of the Composition Microorganisms of the SFL During Ensiling Rice Straw at 10°C

DGGE results showed that the SFL colonized into the fermented system. However, the roles of composition microbes in the fermented system were not clear. For this purpose, quantitative PCR was used.

According to Fig. 6, the DNA mass of A and B increased dramatically during the first six days of fermentation. By sixteen days of fermentation, they had occupied 68% of the microflora DNA mass. As for A, the DNA mass increased continuously until sixteen days of the fermentation, which occupied 65%. For B, the DNA mass amounted to 5.5% at six days of the fermentation, which was the maximum relative value during the fermentation.

DISCUSSION

Sampling in the corresponding environment or area is one of the general methods used to obtain the target microorganism. When the numbers of target microorganisms are lower, enrichment cultivation becomes a way to direct selection [22]. In our laboratory, two enrichment cultures from the corresponding environment, MC1 and A12, had been achieved by direct selection [7, 24]. In this study, a similar method was taken. At the beginning of selection, eleven groups of microflora were enriched or cultivated at 5°C, including inoculants for ensiling at ambient temperature. As the time progressed, most groups were eliminated owing to slower growth and/or decline in pH. Finally, one group from rice straw and soil at a rice field of Northeast China was selected. It indicated that sampling in the low-temperature area can become a key base for selection.

At present, wasting and utilization of straw has resulted in common attentions all over the world. For example, many efforts have been attempted to convert straw into energy. However, there still exist some problems under development [12]. Straw used as feed already has a long history. As a way to convert wastes into products, farmers in Northeast China feed straw as the main diet for ruminants in winter. Previous reports has proved that ensiling was an effective way to improve the palatability of straw [25, 28]. In this study, to investigate the feasibility to ensile straw at low temperature, the SFL was selected and inoculated into rice straw for lab-scale fermentation at 10°C. Compared with the commercial inoculant, the SFL lowered the pH of the fermented rice straw more rapidly and produced more lactic acid. Although the decline in pH and increase in lactic acid were not as distinct as those at 30°C (data not shown), it indicated that ensiling could fasten the fermentation of rice straw to some extent. To the authors' knowledge, this is the first report on ensiling rice straw with the start culture at 10°C.

In this study, the colonization of inoculants was detected using DGGE. Although DGGE does not reflect all members of the detected bacteria [7], it has specific advantage in monitoring the predominant microbial species [5, 11, 17]. According to the profiles of DGGE, the microbial dynamics were similar between the control and the rice straw with the CI, the possible reason being the higher similarity of microbial compositions between the CI and the epiphytic LAB in rice straw. Furthermore, it also indicated that fermentation effects of the CI or the epiphytic LAB at ambient temperature in rice straw were not so good at low temperature. In addition, from the patterns of DGGE, it distinctly suggested that the SFL could colonize into the fermented system.

Construction of a clone library showed that the SFL contained two LAB species. By sixteen days of the

fermentation, they had occupied 68% of the fermentation microflora DNA mass. This result indicated that the SFL became the main part of the microflora during the fermentation. The closest species of the composition microorganisms previously had mostly been reported in meat products during cold storage [4, 16, 20]. There were few reports about their enrichment from soil and/or straw. DGGE revealed they could colonize the fermented system. To investigate the dynamics of the composition microorganisms of the SFL during rice straw fermentation, quantitative PCR was used. The overwhelming DNA mass of species A indicated that it possibly played a key role during the rice straw fermentation at low temperature. To make sure the role of A, the inoculating effects between A and the SFL should be compared. Such experiments are now under way in our laboratory.

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