African Journal of Microbiology Research Vol. 6(36), pp. 6537-6544, 20 September, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.418
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Effect of *Bacillus cereus* Br on bacterial community and gossypol content during fermentation in cottonseed meal

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Accepted 3 September, 2012

To define new methods for the detoxification of free gossypol (FG) within cotton seed meal (CSM), FG during CSM fermentation with *Bacillus. cereus* Br were measured. Four conditions were studied: CSM inoculated with Br (Br + CSM), CSM without any inocula (CSM), sterilized CSM inoculated with Br (Br + sCSM), and sterilized CSM without any inocula (sCSM). Samples were taken at various times during the fermentation and the contents of free gossypol (FG), crude protein (CP), and amino acids were measured. The polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) method was used to analyze the changes in bacterial community structure during the fermentation. After the *Bacillus. cereus* Br was inoculated into CSM, the FG concentrations decreased to the minimum level of detection at 12 h, while CP and amino acids concentrations reached maxima at 18 h. The initial content of FG and CP decreased significantly when the CSM was pre-sterilized, and decreased to minima after 12 h fermentation. The changes in bacterial community structure mirrored the changes in FG and CP. *B. cereus* Br was dominant in the CSM + Br group during the first 12 h of fermentation, after which Br growth was inhibited by native bacteria in CSM. In contrast, *B. cereus* Br was always dominant during fermentation of autoclaved CSM. These results suggest that the native microbes of CSM significantly influenced the detoxification rate, and inhibited *B. cereus* Br growth during fermentation. But pre-sterilization of CSM significantly affects the FG content and quality of CSM. The *B. cereus* Br was capable of detoxifying FG from CSM, and the optimal time of fermentation was 18 h.

Key words: Bacterial community, free gossypol, detoxification, fermentation, cottonseed meal.

INTRODUCTION

Cottonseed meal (CSM) is a source of high-quality protein. The use of this nutrient rich resource as animal feed, however, is hampered by the presence of free gossypol (FG), a toxic polyphenolic pigment produced in the seeds. Diets containing FG can negatively effect animal growth, digestive health, and reproduction (Santos et al., 2003; Carruthers et al., 2007; Cai et al., 2011; El-Saidy and Saad, 2011; Özdoğan et al., 2012; Zheng et al., 2012). Methods have been developed to detoxify FG from cottonseed, such as solvent extraction (Cherry and Gray, 1981; Rahma and Rao, 1984; Qian et al., 2010; Saxena et al., 2012), chemical treatment with ferrous sulfate (Barraza et al., 1991; Tabatabai et al., 2002), and calcium hydroxide (Nagalakshmi et al., 2002, 2003). All of these methods are effective in detoxification, but contamination from residual solvents may be potentially harmful to animals. Ferrous sulfate can cause feed to turn black, whereas calcium hydroxide often reduces the biological activity of vitamins and lowers detoxification efficiency (Zhang et al., 2007).

Microbial fermentation might be the best detoxification method, because microorganisms not only reduce the FG, but also improve the feed value of CSM by enhancing the content of protein, free amino acids, and secreted coenzymes such as cellulolytic enzyme, amylase,

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protease, and lipolytic enzyme, in addition to some vitamins and other unknown active substances (Wu and Chen, 1989; Shi et al., 1998). Some microorganisms, such as *Geotrichum candidum*, *Candida tropicalis, Torulopsis candida, Aspergillus flavus, Aspergillus niger* and *Aspergillus oryzae* have been shown to degrade FG, and so may reduce FG during solid substrate fermentation (SSF) (Zhang et al., 2007; Khalaf and Meleigy, 2008; Sun et al., 2008; Lim and Lee, 2011; Yang et al., 2011, 2012). In almost all previous studies, the CSM was pre-sterilized by autoclaving before being used as the basal substrate in SSF. High temperature treatment alone would significantly reduce the FG content, so the rate of detoxification attributable to the microorganisms is unclear. Furthermore, pre-sterilization of CSM and maintenance of a sterile environment would be expensive for mass production. The native microbes in unsterilized CSM would also undoubtedly influence the microbial detoxification in SSF. However, there are few studies examining how this microbial community change affects the detoxification of CSM during the fermentation process. In the current study, *B. cereus* Br was used in CSM fermentation. We analyzed the composition of the bacterial communities during fermentation of either untreated or pre-sterilized CSM, and measured the levels of crude protein, free amino acids, and FG at specific times during fermentation.

MATERIALS AND METHODS

Basal substrate treatment and microorganisms

The CSM was obtained from Tai-kun Corp. (Xinjiang, China) and stored at room temperature (25 to 30°C) until used. About 2.5 kg CSM was packaged with brown paper and autoclaved at 121°C for 30 min. The strain *B. cereus* Br was used in this study. It was isolated from the soil of cotton fields and is capable of utilizing FG as a carbon source. The inocula were prepared by inoculating *B. cereus* Br into a 500 ml Erlenmeyer flask containing 100 ml of sterile LB medium. The Erlenmeyer flask was incubated on a rotary shaker at 200 rpm for 16 h at 37°C.

Solid substrate fermentation with *B. cereus* Br and sampling

The CSM was moistened with distilled water at a ratio of 1:0.8 (w/w). There were 4 treatment groups in the experiment: CSM inoculated with *B. cereus* Br (Br + CSM), CSM without inocula (CSM), sterilized CSM inoculated with Br (Br + sCSM), and sterilized CSM without any inocula (sCSM). After blending, the mixtures were put into a sterilized plastic drum, and then incubated at 30°C for 48 h under 85% relative humidity. All treatments were repeated in triplicate. About 30 g samples were taken at 0, 6, 12, 18, 24 and 48 h during fermentation. All samples were put into sterile bags and stored at -70°C for further treatment and analysis.

Sample processing

Fermented substrates were thawed, and about 1 g of each sample was placed into sterile 10 ml centrifuge tubes for total DNA extraction. The rest of the fermented substrates were dried in an oven at 55°C for 48 h and subsequently processed into flour for other tests.

Total DNA extraction from fermented CSM

Before extraction of DNA, about 5 ml decolor buffer (100 mM Tris-Cl, 50 mM EDTA, 100 mM NaCl, 0.01 mM PVP, 0.02 mM Na_2CO_3, pH 10) were added into the centrifuge tube containing the fermented CSM. After vortexing for 2 min, the tubes were placed in a 37°C water bath for 10 min, vortexed again for 2 min, and then centrifuged at 9000 × g for 10 min. The supernatant was gently removed using a pipette tip and discarded. These steps were repeated 3 or 4 times until the supernatant was clear. Then 2 ml DNA extraction buffer (2% w/v) CTAB, 1 mM NaCl, 50 mM EDTA, 50 mM Tris-Cl, pH 8.0) containing lysozyme (4 mg/L) was added, and the tubes incubated at 37°C for 1 h. Next, 2 mg/L proteinase K (Merck Corp.) and 1% sodium dodecyl sulfate were added and the mixture incubated at 55°C for 2 h. Release and precipitation of genomic DNA was done using the method of Yeates et al. (1998). The total DNA extracted from the fermented CSM was purified with a Spin Column DNA Gel Extraction Kit (Sangon Corp.) using the manufacturer's recommendations. Isolates were stored at -20°C until analyzed.

PCR and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments

The DNA extracts were used as templates for PCR amplification of the bacterial 16S rDNA gene fragment with primers 341F-GC (the complement of EUB341 with a 40-bp GC clamp 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GCCTACG GGAGGCAGCAG-3') and 517R (the complement of EUB517 5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). A "touchdown PCR" (Don et al., 1991) was used according to the protocol of Riemann et al. (1999).

The PCR products were purified with a Spin Column DNA Gel Extraction Kit (Sangon Corp.) and the concentrations of PCR products determined by NanoDrop 2000 (Thermo Corp.). Equal amounts of PCR products were loaded on 8% polyacrylamide gels containing a denaturant gradient of 40 to 60% from top to bottom, where 100% is defined as 7 M urea and 40% (v/v) formamide. Denaturing gradient gel electrophoresis was performed with a D-code universal mutation detection system (Bio-Rad Corp.) at 60°C for 6 h at 150 V in 1×TAE buffer. The gels were stained for 20 min in 1×TAE containing 0.5 mg/L ethidium bromide and then washed for 15 min in distilled water. Gel band profiles were inspected under UV illumination.

Dominant bands were excised from DGGE gels, and DNA was eluted overnight in 50 µl TE buffer at 4°C. Then, 16s rDNA gene fragments were re-amplified from excised bands, and analyzed by a second DGGE in order to ensure that the relevant bands were resolved. The PCR products were purified using a Spin Column DNA Gel Extraction Kit (Sangon Corp.) and ligated into the PMD18-T vector (Takara). The ligation products were transformed into *Escherichia coli* DH5α competent cells and 3 clones with a 200 bp insert from each band was sent to Sangon Corp. for sequencing. The sequences were then aligned with closely related 16s rDNA sequences in GenBank, and a phylogenetic tree was constructed using MEGA 4.1 software.

The DGGE profiles were analyzed using Quantity One 1-D software (Bio-Rad Corp.) to determine the position and intensities of individual bands. The DGGE profiles of bacterial communities were analyzed by subtracting the background fluorescence from each lane, and then band intensities were normalized to the total intensity of all bands in a given lane to give relative band intensities. Different lanes and their relevant band intensities constituted a

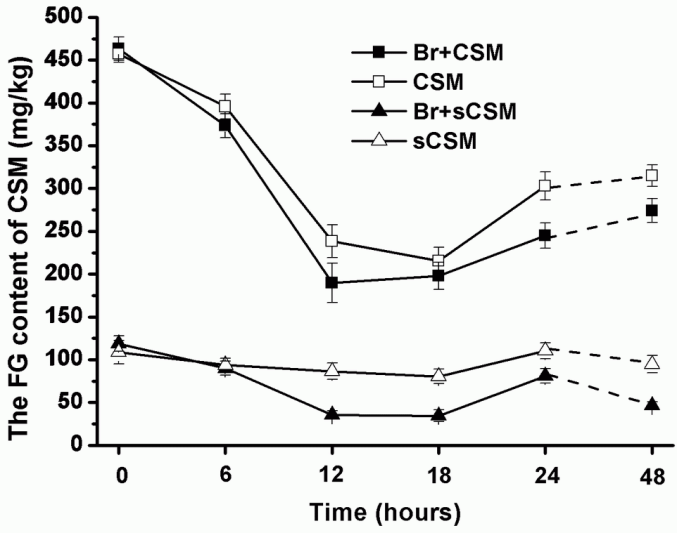

Figure 1. The FG concentration during the CSM fermentation under the four conditions. *Legend*: Br+CSM: treatment of CSM inoculated with *B. cereus* Br. CSM: treatment of CSM without inocula. Br + sCSM: treatment of sterilized CSM inoculated with *B. cereus* Br. sCSM: treatment of sterilized CSM without any inocula. Each point represents mean±standard deviation from three independent assays. There are not significantly different (P>0.05) between the results of repeated experiments.

matrix, and Matlab was used to conduct principal component analysis (PCA) of the change of bacterial species during the fermentation of CSM.

Related assays

The dry matter (DM) was used for related assays. The FG content was determined as described by Hron et al. (1996). Crude protein (CP) assays used the Kjeldahl method (AOAC, 1999). Free amino acid assays was based on the AOAC method (1999, method number 994.12) using an Hitachi Model L-8800 automatic amino acids analyzer (Hitachi Corp.).

Statistical analysis

The data from each experiment were pooled and then evaluated by one-way ANOVA (SPSS 13.0 for Windows) and least significant difference (LSD).

RESULTS

Variation of FG concentrations during the CSM fermentation

The FG concentration rapidly decreased from 0 h (463.3 mg/kg) to 12 h (189.7 mg/kg) during the fermentation after *B. cereus* Br was inoculated into CSM (Figure 1). The maximum detoxification was 59.1%. Then the FG concentration began to increase to 274 mg/kg by the 48th hour. The FG concentration in CSM without any inocula approximately mirrored that of CSM inoculated with *B. cereus* Br, but the lowest FG concentration appeared at 18 h (215.3 mg/kg) for a maximum detoxification of 52.9%. After autoclave sterilization at 121°C for 30 min, the FG content of the CSM was only 113.7 mg/kg (detoxification of 75.3%). The lowest FG content of the inoculated autoclaved CSM (Br + sCMS) appeared at 18 h (34.3 mg/kg DM, 71% detoxification).

Variation of crude protein and amino acids concentrations during the CSM fermentation

The amounts of crude protein during the CSM fermentation are shown in Figure 2. The CP content of the fermented CSM increased from the 6th hour to the 18th hour, after which it began to decrease. The CP content of the CSM was enhanced by 3.3%, whilst the CP content of uninoculated sCSM was enhanced by 3.5%. The increased velocity of CP accumulation in the fermented CSM inoculated with *B. cereus* was only slightly higher than the un-inoculated group during 0 to 12 h. Autoclaving lowered the CP content of the CSM ($P < 0.05$) in the inoculated CSM at all sample time points. The consumption of carbohydrate by microbes during the fermentation, or the release of volatile materials during treatment, could cause the relative increase in CP content in the unsterilized fermented CSM. These results indicate that pre-sterilization before fermentation with Br leads to a significant loss of nitrogenous materials and poorer initial quality CSM.

Total amino acid reached a maximum at the 18th hour in the Br + CSM condition, and then began to decrease with continued fermentation (Table 1). The essential amino acids had the same tendency; levels of lysine increased by 16.4 and 15.3% by the 18th hour in the CSM and Br + CSM groups respectively. Autoclaving lowered the total amino acids content of the CSM (as well as the CP content), and the total amino acid content continued to decrease, reaching a minimum at 18 h. However, the total amino acid content of the autoclaved CSM without any inocula remained statistically unchanged ($P > 0.05$).

Change in bacterial community structure during the CSM fermentation

The PCR-DGGE method was used to analyze the variation in bacterial community structure, and the results showed that the bacterial populations changed significantly in the CSM and Br + CSM groups during fermentation (Figure 3). In the DGGE profile, the intensity of bands 5 and 8 increased from 0 to 12 h, and decreased after 18 h. This suggested that the strains indicated by bands 5 and 8 were dominant in the bacterial community. However, the bacteria species represented by band 5 was always dominant during the fermentation of sterile CSM with Br (Br + sCSM group,

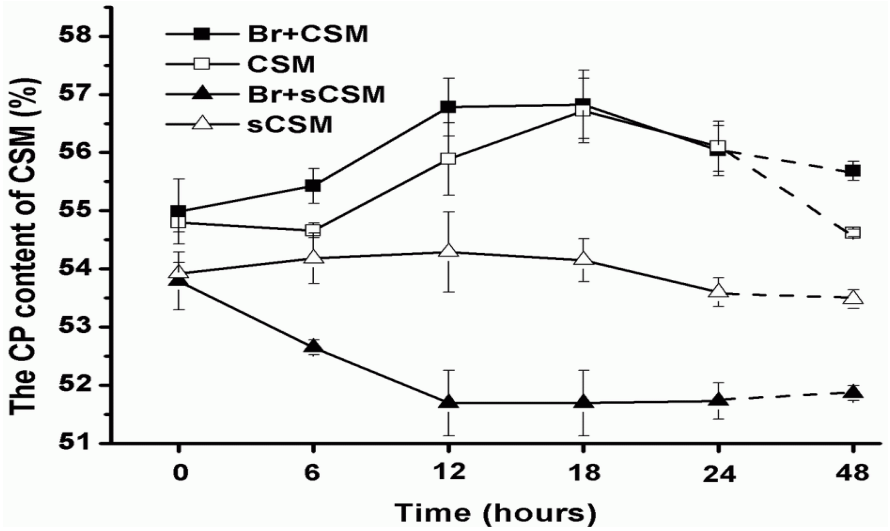

Figure 2. The crude protein concentrations during the CSM fermentation. *Legend*: Br + CSM: treatment of CSM inoculated with *B. cereus* Br. CSM: treatment of CSM without inocula. Br + sCSM: treatment of sterilized CSM inoculated with *B. cereus* Br. sCSM: treatment of sterilized CSM without any inocula. Each point represents mean ± standard deviation from three independent assays. There are not significantly different (P>0.05) between the results of repeated experiments.

Table 1. The amino acid concentrations (g kg^{-1} DM) during the CSM fermentation[a].

Amino	Br + CSM			CSM			Br + sCSM			sCSM		
acids	0 h	18 h	24 h	0 h	18 h	24 h	0 h	18 h	24 h	0 h	18 h	24 h
ASP	46.8	48.5	48.4	47.1	48.7	48.1	44.9	44.0	44.2	45.9	46.7	46.5
THR	16.5	17.9	17.2	16.8	17.9	16.9	16.5	16.5	16.2	16.0	16.6	16.2
SER	22.6	23.1	23.4	22.8	23.4	23.2	22.0	20.7	21.8	21.8	20.4	22.3
GLU	121.5	123.9	124.9	121.7	123.3	123.5	117.8	108.2	116.7	116.5	117.8	118.7
GLY	20.7	21.8	21.3	20.8	21.7	21.1	20.2	19.4	19.6	20.2	20.5	20.4
ALA	20	23.6	20.5	20	22.5	20.3	19.8	20.5	19.5	20.1	20.8	20.2
CYS	8.3	8.8	9.0	9.2	9.0	9.3	8.1	7.6	8.1	8.8	8.6	8.3
VAL	21.8	23.9	22.4	21.9	24.2	22.2	21.7	22.2	21.3	21.9	22.7	22
MET	6.0	6.3	5.8	6.3	6.2	6.6	6.0	5.8	5.4	5.8	5.9	6.0
ILE	15.8	17.4	16.0	15.7	17.4	15.8	15.7	16.4	15.2	15.6	16.1	15.8
LEU	31.2	33.4	31.7	31	33.7	31.2	30.7	30.9	30.5	31.1	31.7	31.7
TYR	12.9	14.0	13.7	13.5	13.9	13.7	13.1	13.0	12.7	13.2	14.4	13.3
PHE	27.1	29.0	27.6	27	28.9	27.4	26.5	25.6	26.2	26.9	27.4	27.4
LYS	18.9	22.0	19.9	18.9	21.8	19.5	19.0	19.2	18.9	19.3	21.4	19.4
NH3	11.0	13.5	11.5	11.1	12.6	11.3	12.1	13.4	11.5	11.5	12.5	11.8
HIS	13.1	13.4	13.4	13.1	13.5	13.3	12.6	12.3	12.4	12.7	12.6	12.9
ARG	59.6	54.3	62.3	60.3	53.5	61.9	54.2	47.6	54.4	58.5	50.4	59.7
PRO	14.0	14.9	14.5	13.9	15.1	14.6	13.7	13.4	13.8	14.3	15.3	14.5
Total	487.8	509.6	503.1	491.2	507.5	500.1	474.6	456.7	468.2	480.3	481.7	487.1

[a] Values are means of three replicates of each samples got on different time per treatments.

Figure 3).

Fourteen main bands were recovered, cloned and sequenced. The sequences were aligned with closely related 16S rDNA sequences from GenBank, and a phylogenetic tree was constructed (Figure 4). The bacteria in the fermented CSM were mainly *Acinetobacter* sp., *Enterobacter* sp., and *Bacillus* sp. Band 9 was the fragment of eukaryotic chloroplast 16S

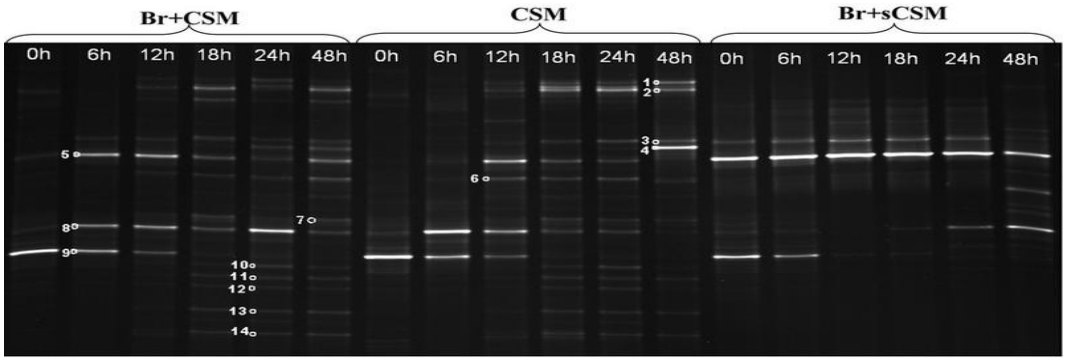

Figure 3. DGGE profile of 16S rDNA gene fragments of bacteria during the CSM fermentation. The group of Br + CSM, CSM and Br + sCSM was the treatment of CSM inoculated with *B. cereus* Br, CSM without inocula and sterilized CSM inoculated with *B. cereus* Br, respectively. The numbers on the top of each lane indicate the time of sampling. All marked bands were analyzed by sequence analysis.

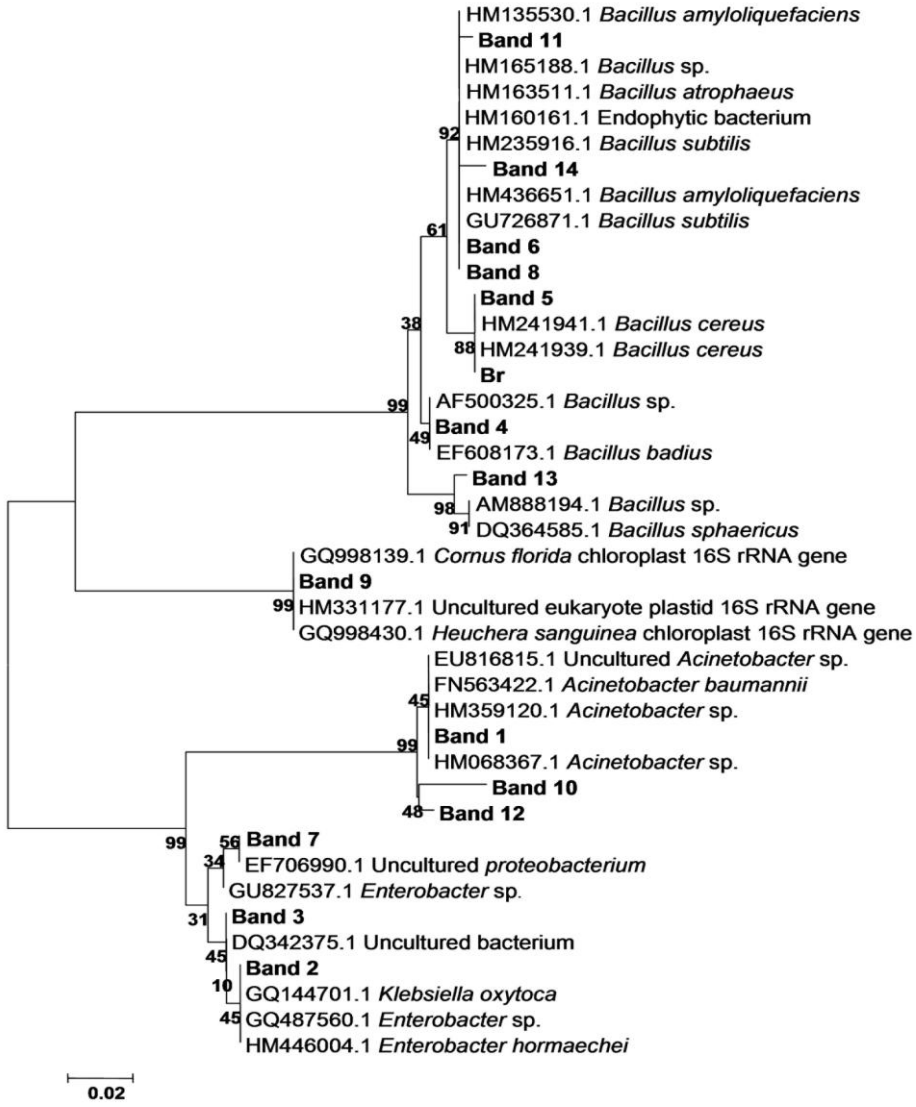

Figure 4. Phylogenetic tree of 16S rRNA gene sequences obtained by PCR-DGGE. Distances were calculated using the maximum-likelihood method, and the tree was constructed using neighbor-jonining method.

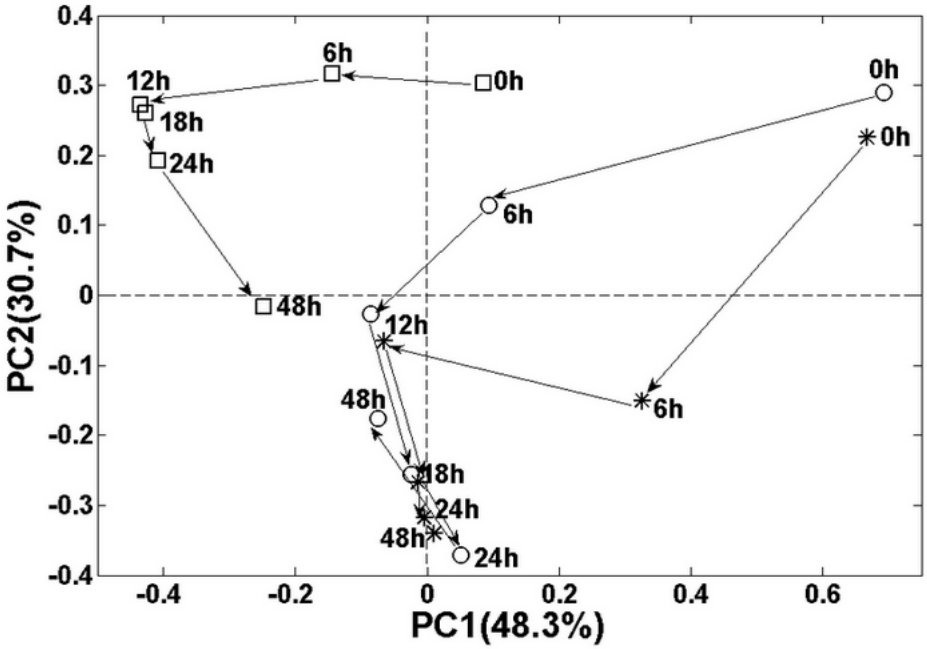

Figure 5. Principal component analysis (PCA) of the DGGE profiles shown in Figure 3. Only the first principal component (PC1) and the second principal component (PC2) are shown. The plots (o, *, and squares) indicate the bacterial community structure of samples from groups Br + CSM, CSM and Br + sCSM respectively. The numbers indicate the time of sampling.

rRNA gene in the fermented CSM. Based on the evidence from sequence alignment, the *B. cereus* Br 16S rRNA gene product was band 5. The bacteria band 8 was most similar to *Bacillus amyloliquefaciens*, the native autochthonal bacteria in CSM.

After analysis and normalization using Quantity One 1-D software, PCA of the DGGE profile was conducted by Matlab. Two principal components (PCs) explained 79% of the total variability in bacterial community structure. Analysis showed that the bacterial communities gradually and continuously changed during CSM fermentation. There were four stages of change in the bacterial structure, rapid changes in stage 1 (0 to 6 h), stage 2 (6 to 12 h), and stage 3 (12 to 18 h). Structures remained stable after 18 h (stage 4). There were three stages during fermentation of autoclaved CSM inoculated with *B. cereus* Br, a rapidly changing stage 1 (0 to 12 h), a stationary stage 2 (12 to 24 h), and a moderately changing stage 3 (24 to 48 h) (Figure 5). The change in bacterial community structure mirrored the changes in FG and CP, suggesting that the bacteria were responsible for the changes in FG and CP during fermentation.

DISCUSSION

Microbial fermentation might be the most efficient method for detoxification of FG from CSM. In most studies of microbial fermentation of CSM, to prevent the effect of native bacteria, the CSM was pre-autoclaved. Zhang et al. (2006, 2007) reported a detoxification rate of 94.6% when autoclaved CSM was fermented by *C. tropicalis*. The authors proposed that heat treatment facilitated microbial fermentation and detoxification of FG in CSM. However, Jia et al. (2009) concluded that detoxification was mainly a result of CSM sterilization, and that the actual detoxification rate for *Candida* sp. was 5.96 to 19.64%. The current work demonstrated a detoxification rate of 75.3% from autoclaved CSM. The reason that the FG concentration was significantly reduced by pre-sterilization of CSM might be the formation of stable bonds between FG and proteins or amino acids in CSM (Nagalakshmi et al., 2002). Furthermore, the fermentation conditions used in the studies mentioned above might be unsuitable for use in mass production because of the huge additional cost. Microbes native to CSM obviously influence the fermentation and detoxification, but little work had been done on how these inherent microbes affect the process of fermentation for detoxification.

The PCR-DGGE method is a useful molecular tool for analysis of bacterial diversity and community structure (Muyzer and Smalla, 1998), and has been used for bacterial community variation analysis in SSF (Guan et al., 2012; Lv et al., 2012). In the present study, the DGGE profile exhibited distinct changes in the bacterial community during fermentation. *B. cereus* Br was the dominant microbe in the fermentation of the autoclaved CSM, and it was dominant before 12 h in the fermentation

of un-treated CSM, which suggested *B. cereus* Br played key role during the fermentation. However, the native bacteria of CSM began to grow rapidly after 12 h so that *B. cereus* Br was no longer dominant, suggesting that the growth of *B. cereus* Br was inhibited by native bacteria. The bacteria band 8 came from native CSM bacteria and belonged to *Bacillus* sp. The species played a similar role to *B. cereus* Br during fermentation. The PCA results showed the variations in *B. cereus* Br and the bacteria represented by band 8 were the principal factors that caused the changes in the constituents measured. Indeed, the different stages in changing bacterial community structure mirrored changes in FG and CP, which suggested that *B. cereus* Br and the inherent bacteria were responsible for the fermentation and detoxification.

It has been reported that some microorganisms are capable of FG detoxification from CSM, including *C. tropicalis, T. candida, A. flavus* and *A. niger* (Weng and Sun, 2006; Zhang et al., 2007; Khalaf and Meleigy, 2008; Lim and Lee, 2011; Yang et al., 2011, 2012), but little work has been done with bacteria. The mechanism of FG detoxification by microbial fermentation remains unclear. It might be caused by transformation of FG to BG (Reiser and Fu, 1962; Kornegay et al., 1972; Jia et al., 2009), or FG may be degraded by the microorganism (Weng and Sun, 2006). In previous studies, it was found that *B. cereus* Br could grow well with gossypol serving as the only carbon source. The FG concentration in the sterilized CSM decreased after the *B. cereus* Br was inoculated, and the detoxification rate in the CSM inoculated with *B. cereus* Br was significant higher than that in the CSM without any inoculates ($P < 0.05$). These results provided evidence that *B. cereus* Br has the capability to degrade FG in CSM.

Microbial fermentation of CSM provided an economic and efficient method not only to reduce the FG levels to an acceptable range, but also to improve the feed value. Undoubtedly, it was very important to reduce the cost while this method was used in mass production. In most of the previous studies, the CSM was pre-autoclaved before fungi were inoculated for fermentation, and the fermentation times were all more than 36 h (Weng and Sun, 2006; Zhang et al., 2007; Khalaf and Meleigy, 2008; Lim and Lee, 2011). Those methods would obviously increase energy and time costs. In the current study, *B. cereus* Br was used to ferment the unsterilized CSM, the FG levels were decreasing in 18 h, and the content of CP and amino acids were increasing. It suggested that the strain *B. cereus* Br had great potential application in mass production of microbial fermentation of CSM.

The interactions between inoculated *B. cereus* Br and the native bacteria would cause complex change in bacterial community during the fermentation in CSM, and which would affect the detoxification (Figure 1). The CSM used in this study was obtained from Tai-kun Corp. (Xinjiang, China); its native bacteria significantly influenced detoxification, and inhibited the growth of the inoculated stain after 12 to 18 h of fermentation (Figure 3). It could not draw hasty conclusions that the same results would get while other CSM were used as substrates for fermentation. However, the present study provided a direction for the microbial fermentation of CSM. And further study of changes in bacterial community structure, and the effect of bacteria community structure on FG detoxification in unsterilized CSM during fermentation, might benefit the application of this method for mass production.

ACKNOWLEDGEMENT

This research was supported by the major projects for science and technology of Zhejiang province [No. 2006C12097-(1), 2011C12010], and in part by a grant for innovative research from Zhejiang Academy of Agricultural Science.

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