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Microbial diversity and dynamics of microbial communities during back-slop soaking of soybeans as determined by PCR–DGGE and molecular cloning

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Abstract Tempe is a traditional fermented food in Indonesia. The manufacture process is quite complex, which comprises two stages, preparatory soaking of soybeans and fungal solid state fermentation. Daily addition of previous soak water (back-slopping) during the soybean soaking step is considered to be crucial in the manufacture of high quality tempe. The microbial diversity and dynamics of the microbial communities evolving during back-slop soaking of soybeans for tempe making was investigated by culture-independent PCR–DGGE and molecular cloning. Both DNA and total RNA were isolated and included in this study, to obtain a view on the succession of total and viable bacteria in the complex microbiota. DGGE profiles indicated that *Enterobacter* sp., *Enterococcus* sp., *Pseudomonas putida*, *Leuconostoc fallax*, *Pediococcus pentosaceus*, and *Weissella cibaria*, were the predominant bacteria. Their occurrence shifted dramatically during the back-slop soaking procedure. This study combined with previous culture-dependent studies could gain a better understanding of the complex microbiota of traditional fermented food and give useful information for its quality control.

Keywords Tempe · Back-slop soaking · Microbial communities · PCR–DGGE

Introduction

Tempe is a traditional fermented food in Indonesia and known for its attractive flavor, texture and superior digestibility. With its high protein content, it serves as a tasty protein complement to starchy staple foods such as rice, so that it can partially substitute meat, eggs or fish, especially for the citizens with poor economic positions. Outside Indonesia, tempe has become increasingly popular as a nutritious non-meat protein food, for example in the USA, the Netherlands and Japan.

The manufacture process of tempe was summarized by Nout and Kiers previously, which comprises two fermentation stages, including a fermentation dominated by a natural bacterial microbiota during the preparatory soaking of soybeans and a fungal solid state fermentation stage, usually with *Rhizopus oligosporus* (Han and Nout 2000; Nout and Kiers 2005). It has been reported that acidification during soaking is favorable for quality and shelf-life of tempe (Nout and Rombouts 1990). Mulyowidarso et al. (1991) demonstrated that lactic acid bacteria dominate during the soaking stage of the traditional process. In principle, a significant lactic fermentation during the soaking stage contributes to low levels of pathogenic and spoilage microorganisms in tempe, which contributes greatly to the safety of this food.

In order to enhance acidification, a ‘back-slop’ procedure, adding previous soybean soak water for next step, is considered to be effective (Nout et al. 1987). As a result of successive back-slops, there is a significant decrease of pH of the soak water, which may result from the effect of bacteria. The microbiota in such soak water communities consisted of complex bacteria, accompanied by a minority of yeasts, which were reported using culture-dependent methods and biochemical identification criteria (Nout et al.

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1987). However, this classical culture-dependent approach only reveals the culturable microbes, numerous other microbiota which may be quite crucial for this process were ignored. Therefore, molecular microbiological methods such as analysis of direct DNA extracts following by PCR-T/DGGE, pyrosequencing, sequencing of clone libraries, etc. are useful additions to realize rapid and high throughput microbial communities analysis, which may greatly develop our knowledge about the actual modification of such special fermentation. In the present investigation we used culture-independent PCR–DGGE and cloning to determine the microbial diversity and microbial community dynamics during back-slop soaking up to 20 back-slop cycles of 24 h each. Both DNA and total RNA were isolated and included in our analysis, to obtain a view on the succession of total and viable bacteria in the complex microbiota.

Materials and methods

Soak water sampling

In order to ensure sufficient acidification, a lactic acid bacteria starter in soybean soak water was enriched by inoculation of soak water with 2.5 % of the soak water of a previous production (back-slopping). One hundred grams dry abrasion-dehulled soybeans were soaked in 500 mL tapwater on the first day, at 30 °C for 24 h. The soaking water was Day 1 sample, 2.5 % of which was added into 100 g soybeans and 500 mL fresh tapwater on the second day. After incubation for 24 h at 30 °C, the soaking water sample for Day 2 was obtained. The other samples (Day 3–20) could be obtained in the same manner. Figure 1 showed the whole back-slopping and sampling process. In the meantime, pH and titratable acidity of each sample were measured every day in order to monitor the process.

DNA extraction

Five hundred microliters soaking water was added into the Lysing Matrix tube which was supplied by FastDNA SPIN Kit for Soil (MP Biomedicals, USA). Total DNA was extracted according to the manufacturer's instructions. DNA concentration and purity were determined by a spectrophotometer (UV–VIS, SHIMADZU, Japan) and finally diluted to 50 ng/μL.

RNA extraction and cDNA synthesis

Five hundred microliters soaking water was treated by RNA Later (Sigma, USA). Extraction was performed by RNeasy Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions. All tubes and tips used in this step were treated with 0.05 % DEPC (diethylpyrocarbonate) previously.

Three hundred nanograms RNA was taken for cDNA synthesis and added up to 11 μL with RNase free water. 1 μL Random primers and 1 μL dNTPmix (10 mM) were added. The mixture was heated at 65 °C for 5 min and incubated on ice for 1 min afterwards. Centrifuged briefly and added 7 μL of Mix buffer (5× First-Stand Buffer 4 μL; DTT (0.1 M) 1 μL; RNaseOUT 1 μL; Superscript III RT 1 μL, all from Invitrogen, USA). The mixture was incubated at 25 °C for 5 min, then 50 °C for 60 min, followed by 70 °C for 15 min. Then the cDNA could be used as PCR template after dilution.

PCR amplification

To investigate the dominant bacterial communities by DGGE analysis, the prepared total DNA and cDNA from soak water was used as a template to amplify the V6–V8 region of the bacterial 16S rDNA gene by PCR, using the primers EUB968-GC- for (5'-AACGCGAAGAACCTT AC-3') with a GC clamp sequence at its 5' end (CGCCC

Fig. 1 Back-slopping process and sampling during soybean soaking stage for tempe making

GGGGCGCGCCCCGGGGCGGGGCGGGGCAGGGG) and EUB-L1401-rev (5'-CGGTGTGTACAAGACCC-3') (500 bp) (Nübel et al. 1996). PCR mixtures consisted of 1×PCR buffer, 0.2 mM dNTP mix, 1.5 mM MgCl₂, 1 μM each primer, and 1.25 U Taq DNA polymerase (Fermentas, USA). 1 μL genomic DNA was added as template. PCR amplification was carried out in a final volume of 50 μL. The conditions were as follows: initial denaturation of DNA for 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 20 s at 56 °C, and 1 min at 72 °C; and extension of incomplete products for 7 min at 72 °C. Amplification products were analyzed by gel electrophoresis.

DGGE analysis

The DCode System (Bio-Rad, Hercules, CA, USA) was used for DGGE analysis, essentially as described previously (Muyzer et al. 1993). PCR products were applied directly onto 8 % (wt/vol) polyacrylamide gels (acrylamide-bisacrylamide, 37.5:1) in a running buffer containing 0.5× TAE (50× TAE stock solution consisting in 2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA [pH 8.0]). Optimal separation of PCR products was achieved with a denaturing gradient of 30–60 % urea and formamide for bacteria, increasing in the electrophoretic run direction. A 100 % denaturant corresponded to 7 M urea and 40 % formamide. Electrophoresis was performed at a voltage of 200 V for 10 min, and then changed to a constant voltage of 85 V for 16 h at 60 °C (Randazzo et al. 2002). The DNA bands were visualized by silver staining and developed as previously described (Sanguinetti et al. 1994).

Cloning and sequence analysis of 16S rDNA in plasmid inserts

Clone libraries of the 16S rDNA amplicons from total DNA or cDNA of soak water were constructed. 16S rDNA was amplified using primers B-for (5'-AGAGTTTGATCC TGGCTCAG-3') and B-rev (5'-AAGGAGGTGATCCA GCCGCA-3') (approximately 1,500 bp) (Niemann et al. 1997). PCR mixtures were set up as described above and amplified under the following conditions: 5 min at 95 °C; 20 cycles, each consisting of 20 s at 95 °C, 20 s at 56 °C, and 1 min at 72 °C; and a final extension of 30 min at 72 °C. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Germany). The concentrations of PCR products were determined and diluted to 70 ng/μL, which were cloned in *Escherichia coli* JM109 using the pGEM-T plasmid vector system (Promega, USA) in accordance with the manufacturer's instructions. After the transformed cells had been incubated with isopropylthiogalactoside (IPTG), 96 random white colonies of ampicillin-resistant transformants were transferred with a sterile

toothpick to 50 mL of TE and were boiled for 15 min to lyse the cells (Kang et al. 2009). Subsequently, PCR was performed with universal primers T7 (5'-AATACGACT CACTATAGGG-3') and SP6 (5'-ATTTAGGTGACAC TATAG-3') (Promega Corp.) to check the size of the inserts (approximately 1,500 bp) by using the cell lysates as the template (Zoetendal et al. 1998). The appropriate regions of the 16S rDNA in the cell lysates of transformants were amplified, and their mobilities were compared to the rDNA-derived patterns of soaking water samples by DGGE (data not shown). The clones that produced a single DGGE amplicon with a melting position identical to that of one of the dominant bands in the soaking water DNA patterns were selected for sequence analysis (Randazzo et al. 2002).

The plasmid inserts from the corresponding clones were sequenced by GATC [GATC Biotech (<http://www.gatc-biotech.com>), Germany]. To determine the closest relative of the DNA sequence, a search of the GenBank database was conducted using the BLAST algorithm (Zhang et al. 2000).

Results

pH and titratable acidity

During tempe fermentation, the back-slop procedure, adding previous soybean soak water for next step, was considered to be effective for acidification. In order to monitor the process, pH and titratable acidity of the 'back-slopping' soak water were detected every day. Figure 2 showed the trends of pH and titratable acidity along with the fermentation process. pH value decreased sharply from 6.77 to 4.32 during the first two days (back-slop cycles) and then stabilized around pH 4.1. Meanwhile the titratable acidity increased rapidly from 0.03 to 0.82 % lactic acid and then stayed around 0.83 % lactic acid later.

DNA-derived DGGE profiles

To investigate the diversity and dynamics of the dominating microbial communities during tempe soaking stage fermentation, samples from soaking water of 8 periods (Day 1–5, Day 10, 15 and 20) were analyzed by PCR–DGGE. The appearance and disappearance of bands in the DGGE pattern (Fig. 3) indicated important shifts in the microbial community structure. The individual bands from different places in the DGGE profile were cloned and sequenced for identification, results of which were shown in Table 1. DGGE marker used in this study was a reference for gel quality, not related to the band identification.

Fig. 2 pH and titratable acidity during tempe soaking stage (titratable acidity was expressed as %lactic acid $\pm$ SD for 3 replicates)

Fig. 3 DNA-derived DGGE profile of tempe soaking stage fermentation (M, DGGE marker; D, sample of Day x)

Table 1 Identifications of predominant bands in DGGE patterns of the total bacterial community in back-slop soybean soak water

No.	Closest sequence relative (species)	% Identity	Genbank accession no.
1	<i>Enterobacter</i> sp.	99	DQ855282
2	<i>E. faecium</i> / <i>E. durans</i>	99	GU904687 EU333895
3	<i>E. cloacae</i> / <i>E. hormaechei</i>	99	Y17665 HQ202859
4	<i>P. putida</i>	99	GQ504714
5	<i>L. fallax</i>	100	AY929288
6	<i>P. pentosaceus</i>	99	FM179610
7	<i>W. cibaria</i>	99	AB494723

The DGGE pattern and identification results of different bands showed that at the beginning of the fermentation, the predominant bacteria were *Enterobacter* sp., *Enterococcus faecium*/*Enterococcus durans* and *Enterobacter cloacae*/*Enterobacter hormaechei*, whereas *Pseudomonas putida*

was also detected. *Leuconostoc fallax* appeared from Day 2 and *Pediococcus pentosaceus* appeared from Day 3. With the process of fermentation, *Weissella cibaria* appeared from Day 10. *L. fallax* and *P. pentosaceus* remained present. But *Enterobacter* spp. and *E. faecium*/*E. durans* decreased day by day based on the lighter and lighter bands and they were not detected from Day 10.

Comparison of DNA- and RNA-derived DGGE profiles

DGGE profiles derived from DNA and cDNA of soak water were compared in order to determine the viable predominant bacteria, which may reflect their contribution to the fermentation. Figure 4 showed the remarkable differences during the first 5 days and the high similarity after Day 10. At the first day, there were three dominant bands in RNA-derived profiles and almost no overlap bands between DNA- and RNA-derived DGGE profiles. Unfortunately, cDNA synthesized by reverse transcription was hard to be cloned, which was different from that of DNA. No identifications of the three bands were obtained from RNA. On Day 3 and 4, RNA-derived profiles gave fewer bands than DNA. Only amplicons of *P. pentosaceus* (band 6) on Day 3 and 5, as well as *Enterobacter* sp. (band 1) in Day 4 showed similar intensities between DNA- and RNA-derived profiles. Similarly, amplicons of the last 10 days showed similar intensities, indicating that these bacteria were metabolically active.

Discussion

A number of microbiological studies were carried out to examine the stages of the tempe fermentation process (Mulyowidarso et al. 1989; Nout et al. 1987). However, few studies have employed culture-independent approaches to investigate the microbiota involved in this process (Seumahu et al. 2012), especially in the back-slop soaking procedure, which is emerging as an important event in the control of

Fig. 4 Comparison of DNA- and RNA-derived DGGE profiles (M, DGGE marker; Dx, DNA from sample of Day x; Rx, RNA(cDNA) from sample of Day x)

tempe quality. Daily addition of previous soakwater (back-slopping) is a powerful method of obtaining a vigorous lactic fermentation. This ‘accelerated natural lactic fermentation’ results from the gradual selection of ecologically adapted acidifying organisms (Nout and Rombouts 1990).

In the present investigation, biodiversity and dynamics of microbial communities during back-slop soaking procedure in tempe fermentation were revealed by culture-independent PCR–DGGE and cloning, which might be compared with classical enumeration results (Nout et al. 1987). Only Enterobacteriaceae, *Lactobacillus*, and *Bacillus* spp. were detected by culturing method, although no numerical information was reported. Our present DGGE profiles showed the variation of microbiota composition, indicating the appearance and disappearance of bands corresponding with major changes in the structure and diversity of populations.

Three bacterial groups were encountered. At the start of the fermentation, the predominant bacteria were *Enterobacter* sp. and *Enterococcus* sp., whereas *P. putida* was also detected. *L. fallax* appeared from Day 2 and *P. pentosaceus* appeared from Day 3, which might explain the decrease of pH value and the increase of titratable acidity. During the fermentation, *W. cibaria* showed up much later from Day 10. *L. fallax* and *P. pentosaceus* were still present, which would be the reason why pH value and the titratable acidity stabilized late in the fermentation. The persistent low pH also explained why *P. putida* and some *Enterococcus* sp. disappear later. PCR–DGGE showed that the lactic acid bacteria mentioned earlier, were dominant during most of the duration of the back-slop fermentation. Because of competitive inhibition, some relatively acid-sensitive bacteria, such as *P. putida*, would not grow at a low pH level. Likewise, other bacterial contaminants would also be inhibited to some extent.

Based on the comparison of DGGE profiles derived from DNA and cDNA of soaking water, the live predominant bacteria, which might reflect their contribution to the fermentation, were dramatically different from the whole communities at the early stages of soaking. This could be the result of DNA of previous soybean microbiota that had

lost its viability, added to DNA of newly evolved microorganisms during soaking. Later, after 5 days, DNA and cDNA showed similar patterns and intensities, indicating that the DNA detected originated mainly from metabolically active lactic acid bacteria.

Seven species of bacteria were isolated as predominant organisms of soak water, which were also reported in other previous works (Nout et al. 1987). However, species, such as *Lactobacillus* spp., *Klebsiella* spp. and *Streptococcus* spp., were not detected but were reported by other studies (Ashenafi and Busse 1991; Mulyowidarso et al. 1989). These different findings might result from several reasons. Firstly, the microbial composition of soak water was shown to depend on the temperature of soaking. At 20 °C, for example, *Lb. casei* and *Staphylococcus epidermidis* were predominant (Ashenafi and Busse 1991). Secondly, several species of the Enterobacteriaceae family, which might also have contributed to the ecology of fermentation at the start, could not survive during the fermentation at 37 and 30 °C, especially accompanying with pH decrease. Moreover, DGGE analysis suffered from the same drawbacks as all PCR-based community analysis techniques, such as biases from DNA extraction and amplification. DNA extraction methods might be selective for some populations over others. Therefore, the combination with culture-dependent and independent approaches could gain a better understanding of the complex microbiota (Waters et al. 2006).

There is good suggestion that tempe is not the product of a pure culture fermentation with *R. oligosporus*, and that bacterial species are essential components of the fermentation. The acidification of soak water was reported to be an important process step enabling the control of the growth of food-borne pathogens and ensuring the microbiological safety of the final tempe (Mulyowidarso et al. 1989; Nout et al. 1987). In this study, biodiversity and dynamics of microbial communities during back-slop soaking procedure were revealed by culture-independent PCR–DGGE and cloning. Combining with previous culture-dependent studies, a better understanding is obtained of the complex microbiota of traditional fermented food and this will add useful information for their quality control.

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