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Comparison of DNA Extraction Methods for Microbial Community Analysis in Indonesian Tempe Employing Amplified Ribosomal Intergenic Spacer Analysis

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Tempe fermentation involved complex microbial communities which are only revealed partially through culture dependent methods. Culture-independent methods would be potential to unravel this complex microbial fermentation. Appropriate DNA extraction is an essential tool to obtain reliable data from culture independent method. In this study, we employed two commercial DNA extraction methods to find the best one for microbial community characterization employing amplified ribosomal intergenic spacer analysis (ARISA). Our result showed that PowerFood Microbial DNA Isolation Kit-MOBIO (PFMDIK) is an excellent method for microbial DNA extraction from tempe. It gave high quantity and quality of DNA suitable for PCR amplification of 16S-23S rRNA intergenic spacer to yield a diverse and reproducible ARISA profile.

Key words: ARISA profile, DNA extraction, tempe microbial community

INTRODUCTION

Soy bean tempe is a traditional Indonesian fermented food which consists of cooked, dehulled soya beans, bound into solid cake by the growth of a mold, most commonly *Rhizopus oligosporus*. Although microbiological studies of tempe production have focused mostly on the fungi as starter, the growth of other microbial species during the process has been reported also, such as bacteria and yeast (Nout & Kiers 2005; Barus *et al*. 2008). The role of some bacteria in tempe fermentation has been studied but only as a single culture. In fermentation, starter culture may contain large number of variable microorganisms, which may be added to accelerate a fermentation process or as a spontaneous fermentation initiated without the use of a starter inoculum (Holzapfel 2002).

The complex dynamics of the microbiological composition in tempe continuously generating new aspects being discovered including the production of specific enzymes or bioactive compounds produced by microbial in tempe (Nout & Kiers 2005). Development of metagenomic approaches has provided an unprecedented level of access to microbial genome from many different environments making it possible to characterize the phylogenetic and functional diversity of cultured and uncultured microorganism from various biomass of interest without the inherent bias of cultivation (Kakirde *et al*. 2010). These techniques are DNA-based methods and are strongly dependent on the DNA extraction and purification methods. In particular, the application of molecular method to food sample requires stringent extraction and purification strategies that ensure efficient recovery of nucleic acid and removal of the numerous compounds that could inhibits PCR assay (Pinto *et al*. 2007).

An automated method of ribosomal intergenic spaces analysis (ARISA) was developed for the rapid estimation of microbial diversity and composition in environment. This technique based on PCR amplification of the 16S-23S intergenic spacer region in the rRNA operon with a fluorescence-labeled primer. Because of less selection pressure, the 16S-23S rRNA intergenic spacer sequence seems to be more genetically variable and species specific than that of 16S rRNA and 23S rRNA gene. ARISA-PCR fragment, ranging from 400 to 1200 bp were then discriminated and measured by using an automated electrophoresis system (Fisher & Triplett 1999; Liu *et al*. 2008).

The aim of the study was to evaluate and compare two DNA extraction methods for microbial DNA extraction in tempe. This study was intended to minimize the influence of DNA extraction and PCR methods on reproducibility of ARISA profile.

MATERIALS AND METHODS

Sample Treatment. Twenty five gram tempe was homogenized in 225 ml NaCl 0.9% for 1 min. The mixture obtained was centrifuged for 1 min at 800 x g and the supernatant was moved into new tubes and centrifuged

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again at 13,000 x g for 5 min. Supernatant was discarded and the pellet was washed with TE pH 8.0 prior to DNA extraction.

DNA Extraction. Microbial DNA was extracted from tempe with two different commercial kits: Fermentas DNA extraction kit (FDEK) and PowerFood Microbial DNA Isolation kit-MOBIO (PFMDIK) according to the protocol described by manufacturers. The DNA extract were analyzed by electrophoresis on 1% (w/v) agarose gel in TAE buffer and stained with ethidium bromide.

PCR Inhibition Assay. To determine whether PCR inhibitor were present in the DNA extracts, the 16S rRNA gene was amplified using the method described by Marchesi *et al*. (1998). The reaction was carried out in C1000™Thermal Cycler (BIORAD) and amplified DNA was detected by electrophoresis on 1.5% (w/v) agarose gel in TAE buffer. The gel was stained with ethidium bromide.

Determination of DNA Concentration and Purity. DNA concentration was determined by measuring the absorbance at 260 nm. DNA purity was measured by calculating the ratio of absorbance at 260 and 280 nm also 260 and 230 nm. NanoDrop 2000 Spectrophotometer (Thermo Scientific) was used for this analysis.

Automated Ribosomal Intergenic Spacer Analysis (ARISA). Bacterial and fungal DNA from the DNA extract was amplified using primers that amplify the intergenic spacer region (ITSF/ITSReub for bacteria and 2234C and 3126T for fungi; Ranjard *et al.* 2001; Cardinale *et al.* 2004). The 5' end of the ITSReub and 2234C were labeled with phosphoramidite dye HEX (6-carboxy-1,4-dichloro-2,4,5,7-tetra-chlorofluorescein).

PCR reaction condition consisted of an initial denaturation step (94 ^{o}C, 3 min) followed by 30 cycles of 94 ^{o}C for 45 sec, 56,8 ^{o}C for Bacterial ARISA (BARISA) or 60,7 ^{o}C for Fungi ARISA (FARISA) for 1 min and 72 ^{o}C for 2 min, followed by a terminal extention step at 72 ^{o}C for 7 min. ARISA sequencing service was conducted in PT Wilmar Benih Indonesia, Cikarang as described by Cardinale *et al*. 2004. Peak size (bp), height and area were estimated by comparison with the internal size standard LIZ 1200 fragment. All procedures were done in triplicate. Tempe was purchase from one producer All procedures were done in triplicate. Tempe was purchase from one producer in Bogor to analyze triplicate treatment and daily sampling for three days for reproducibility of microbial communities.

RESULTS

DNA Quantity and Quality. Sample treatment such homogenization time and TE buffer washing gave a good result which yielded no significant deviation among triplicate extractions. Size of the food particle was equal and gave the same chance to be extracted by both methods. PFMDIK yielded 2 to 3 times more DNA compared to FDEK. The quality of microbial DNA extract as determined by spectrophotometry (260/280 and 260/230 nm ratio) indicated that PFMDIK yielded higher quality DNA for 260/280 nm ratio but lower quality DNA for 260/230 ratio. In contrast, FDEK yielded low ratio A260/280 nm but higher ratio A 260/230 (Table 1).

Microbial DNA extract derived from PFMDIK could also be observed on agarose gel, although it appeared only as a weak band. On the other hand DNA from FDEK extract was not visible on the gel although the amount loaded was same (100 ng).

PCR Inhibition Assay. PCR of 16S rRNA gene indicated that DNA extract from PFMDIK were suitable for PCR amplification (Figure 1). The low value of A260/230 in PFMDIK derived-microbial DNA extraction did not interfere with PCR reaction. The PCR results appeared as thick bands, that were significantly different from 16S rRNA gene amplicons derived from FDEK DNA extract. The 16S rRNA gene band could be diminished if the FDEK DNA extract used again for an interval time as a template for the next amplification. This might be happened because of the nucleases as an interfering agent degraded the DNA extract.

Automated Ribosomal Intergenic Spacer Analysis (ARISA) Profile. Amplification of intergenic sequences and ARISA from FDEK and PFMDIK yielded slightly different profiles (Figure 2 & 3). Although both ARISA profile (BARISA and FARISA) from FDEK and PFMDIK showed some profiles with the same size, PFMDIK yielded higher diversity (Shonnon Wienner Index) and higher number of ARISA operational taxonomic unit (OTU) compared to FDEK (Table 2). PFMDIK OTU sizes were more diverse than FDEK. Both methods yielded

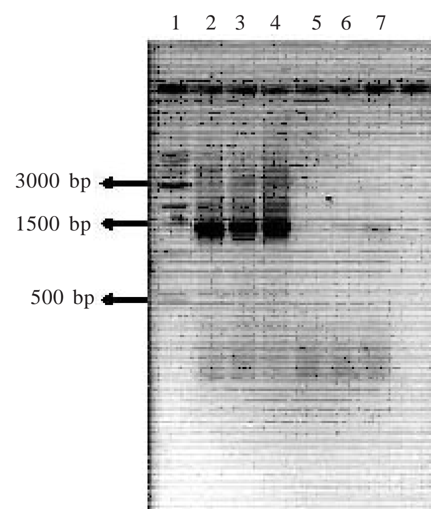

Figure 1. PCR amplification of 16S rRNA genes. Lanes 1. Molecular marker (1 kb ladder), 2. TU1-PFMDIK, 3. TU2-PFMDIK, 4. TU3- PFMDIK, 5. TU1-FDEK, 6. TU2-FDEK, 7. TU3- FDEK.

Table 1. DNA concentration and ratio of A260/280 and A260/230 from two DNA extraction methods

Sampel	Rata-rata kons DNA (ng/µl) ± SD (n=3)	Rata-rata rasio A260/280 ± SD (n=3)	Rata-rata rasio A260/230 ± SD (n=3)
TU- FDEK	3.47 ± 1.55	1.05 ± 0.20	0.95 ± 0.14
TU- PFMDIK	6.9 ± 0.60	1.50 ± 0.03	0.29 ± 0.18

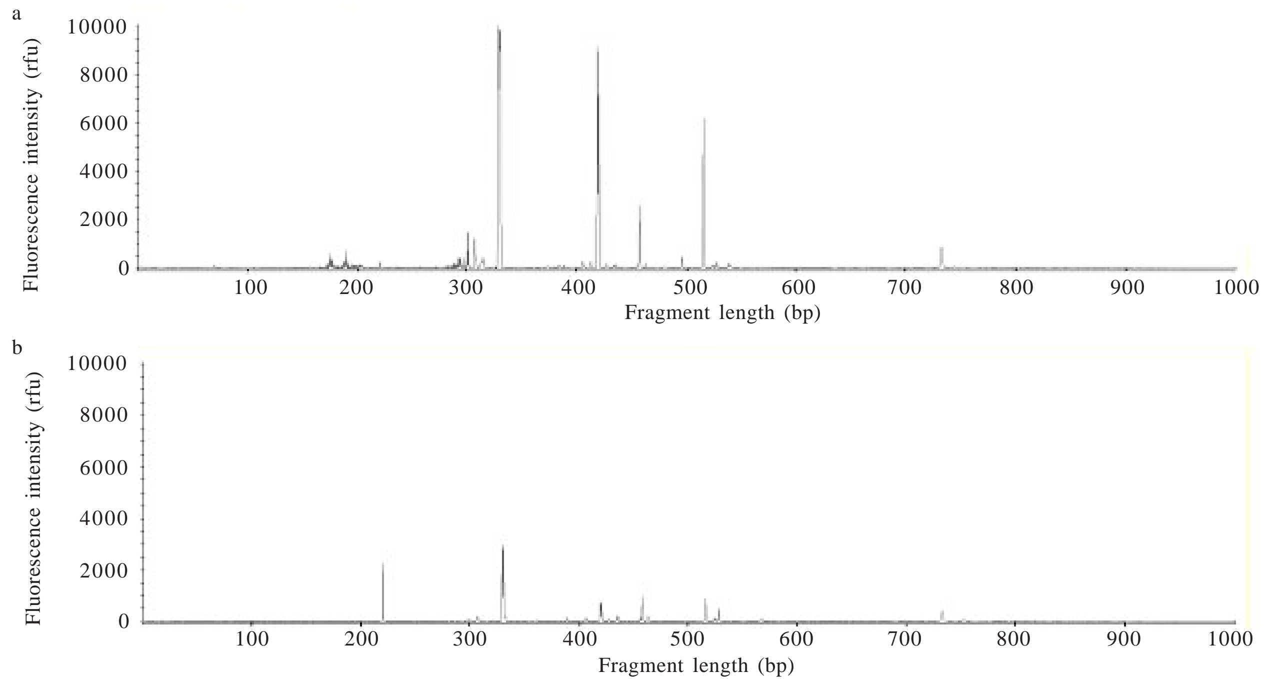

Figure 2. BARISA profiles of the intergenic spacer amplified of two DNA extraction methods: a. PFMDIK, b. FDEK.

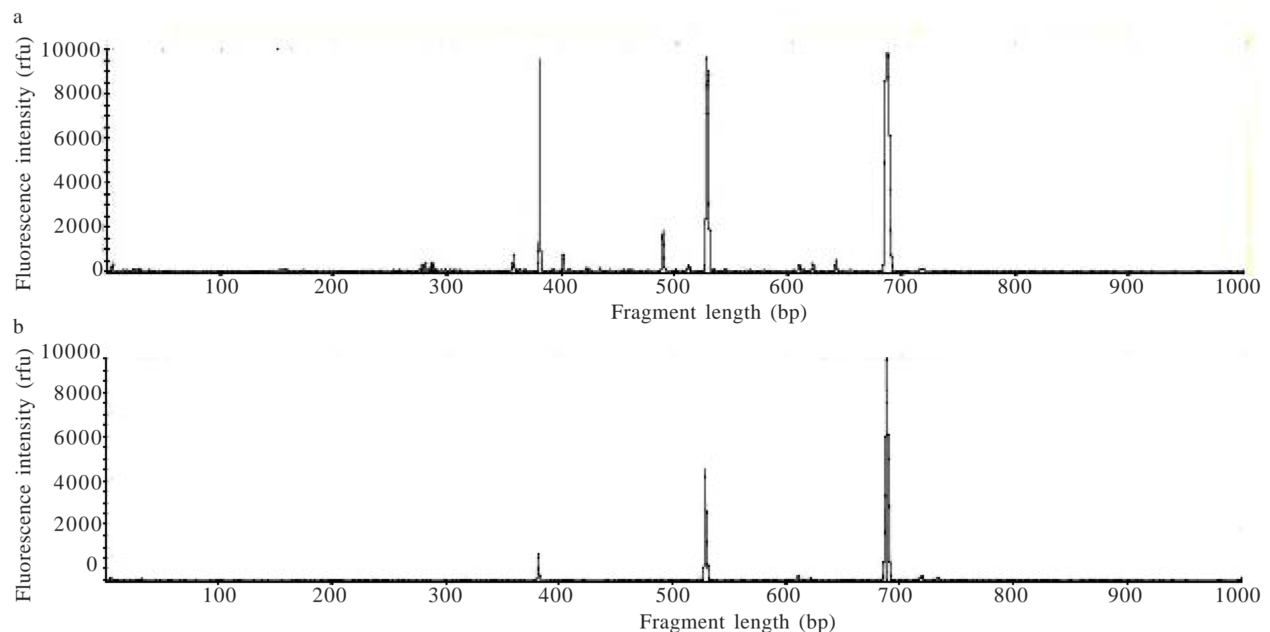

Figure 3. FARISA profiles of the intergenic sequence amplified of two DNA extraction methods: a. PFMDIK, b. FDEK.

Table 2. Comparison of ARISA and BARISA profiles derived from FDEK or PFMDIK extraction

Methods	No. of ARISA OTU		Shannon-wienner index (H)	
	BARISA	FARISA	BARISA	FARISA
FDEK	17	6	0.946	0.510
PFMDIK	79	44	1.036	0.977

reproducible profiles in triplicate replications. However PFMDIK profiles allowed the whole community profile to be better demonstrated than FDEK.

The reproducibility of PFMDIK method was further verified employing tempe sampled everyday for three days from the same tempe producer. The results showed that even the OTUs were rather fluctuate in quantity, the OTUs were still found in higher number and in reproducible profiles; i.e. 83 OTU for BARISA (Figure 4) and 36 OTU for FARISA (Figure 5).

DISCUSSION

Ideally, DNA extraction methods should be simple, quick and efficient, but choosing a good method must involve techniques to yield optimal DNA and the removal of substances that could interfere PCR reactions (Jara *et*

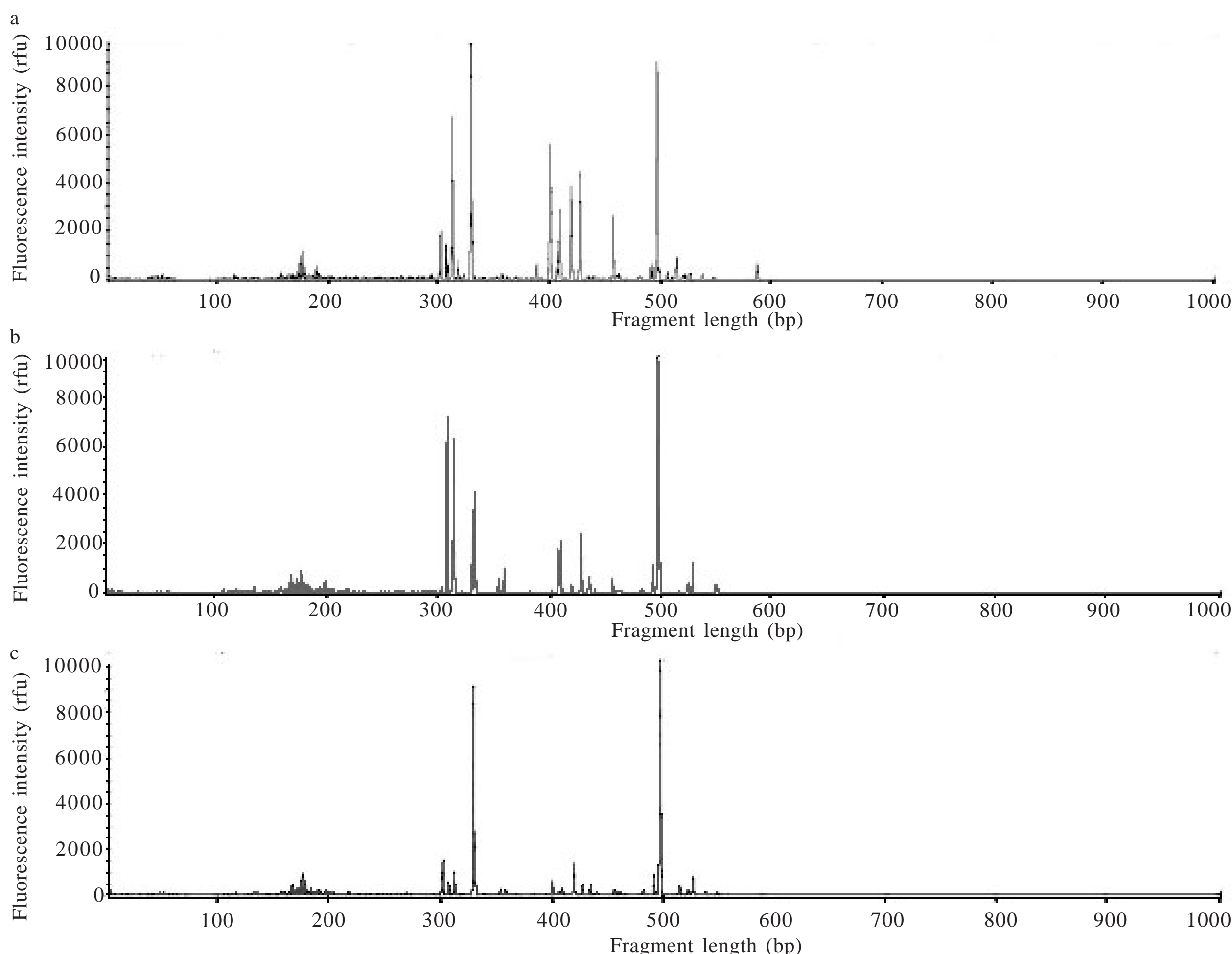

Figure 4. BARISA profiles (triplicate replications a, b, c) of the intergenic sequence amplified from PFMDIK DNA extraction method.

al. 2008). Factors that could inhibit the amplification of nucleic acid by PCR are present with target DNAs from many sources. The inhibitors generally act at one or more of three essential points in the reaction in the following ways: they interfere with the cell lyses necessary for extraction of DNA, promote DNA degradation or capture, or inhibit polymerase activity for amplification of target DNA (Wilson 1997). FDEK yielded low DNA recovery that was reflected in the visualization of genomic DNA on agarose gel and the quantity of genomic DNA as measured by spectroscopy. This result could happen during the lysis step, chemical lyses leading to degradation of extracted DNA by nucleases. Lyses step in PFMDIK employed both chemical and mechanical steps and PCR inhibitory effect was also eluted.

The DNA with low quality might contain inhibitory substances interfering with PCR reaction (Rossen *et al*. 1992). The most obvious origin of PCR inhibitors in endogenous contamination are compounds present in insufficiently purified target DNA. Unavailability of target or primer DNA by nonspecific blocking or sequestration may inhibit amplification or cause misleading band variations during typing based on PCR. Bacterial cell or debris, proteins and polysaccharides that have caused inhibition in many studies may do so by physical effects, such as making the target DNA unavailable to the polymerase. Some nucleases are produced by many bacteria, exhibiting uncommon heat stability and was able to hydrolyze genomic and primer DNA during amplification reaction (Wilson 1997).

The interfering substances might have been removed by PFMDIK method so the PCR amplification yielded a thick band of 16S rRNA genes that appeared on agarose gel in comparison to that derived from FDEK with the ratio of A260/230 in PFMDIK DNA extract might indicate of contaminant that did not appear to interfere with PCR reactions.

This result showed that PFMDIK is a better method to extract microbial genomic DNA and remove the interfering substances from tempe. This is an important step in studying tempe metagenomic since total genomic DNA should reflect or be a representative of total microbies involved in the fermentation processes.

ARISA could be a potential method because it allows monitoring the occurrence of cultured and uncultured-microbes and can be implemented in application requiring high phylogenetic resolution, reproducibility and high throughput (Popa *et al*. 2009). This technique should allow best separation and yield fingerprints of microbial communities which can then be used to compare whole microbial communities of different food environment (Abriouel *et al*. 2006). However this technique depends

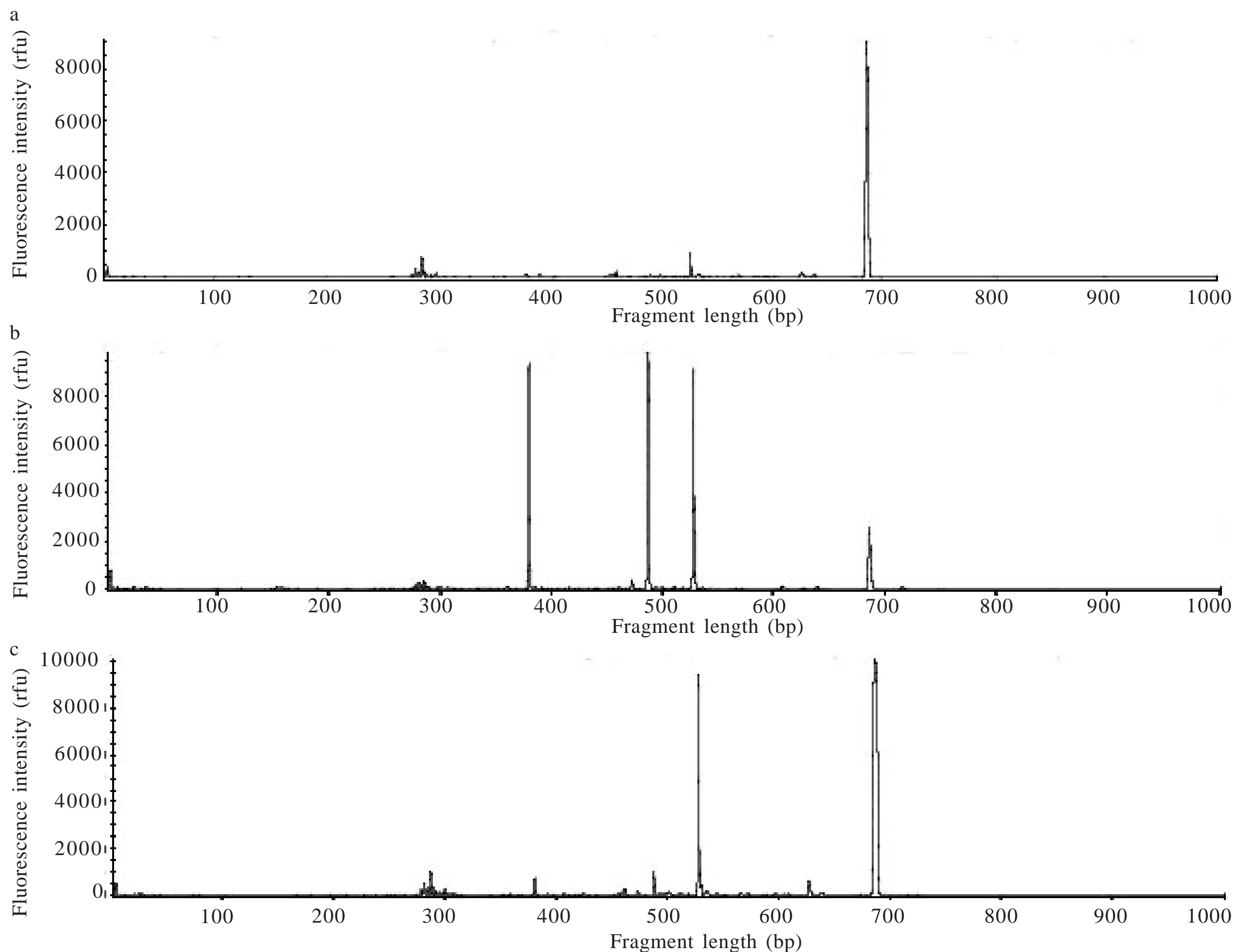

Figure 5. FARISA profiles (triplicate replications a, b, c) of the intergenic sequence amplified from PFMDIK DNA extraction method.

on the extraction and PCR amplification, which should be optimized before conducting ARISA. PFMDIK yielded higher diversity and more reproducible BARISA and FARISA fingerprints compared to FDEK. This method will allow us to explore the microbes involved in the tempe fermentation processes, which could be used as a reliable method to fingerprint or barcode different type of tempe or processes. In conclusion, PFMDIK was suitable for tempe DNA extraction to study microbial community employing ARISA.

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