



Yeast community of Indonesian Tempeh based on ITS-PCR T-RFLP analysis

Efriwati¹, Suwanto A^{2*}, Rahayu G² and Nuraida L³

¹Centre for Biomedical and Basic Health Technology, National Institute of Health Research and Development, Ministry of Health Republic of Indonesia

²Department of Biology, Faculty Mathematics and Natural Sciences, Bogor Agricultural University and asuwanto@indo.net.id

³Department Food Science and Technology, Faculty of Agricultural Technology, Bogor Agricultural University

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Abstract

Tempeh is the most highly consumed traditional fermented food in Indonesia. It is a product of a mixed microbial fermentation which harbors various types of molds, yeasts, and bacteria. Previous studies indicated that tempeh production methods might affect yeast population. However, studies of yeast community structure and population dynamics during tempeh production using DNA directly extracted from tempeh has not been reported. This research aimed to study the effect of two different tempeh production methods (method A and B) on yeast community phylotypes at five stages of tempeh production. The yeast community was estimated by PCR amplification of ITS1- 5.8S rRNA- ITS2 DNA sequence, followed by T-RFLP analyses. Boiled soybean and tempeh at different stages of fermentation was used as a source of metagenomic DNA. The result indicated that many yeast phylotypes were found in all stages of both tempeh production methods. There are two groups of yeast community phylotypes in tempeh produced by method A, and three groups of yeast community phylotypes in tempeh produced by method B. Nine phylotypes were found to be common and two of them predominant in both tempeh samples. Some phylotypes might be specific in one of the stages or in the tempeh production methods.

Key words – diversity – fermentation – phylotypes – predominant – soybean

Introduction

Tempeh has been used as a healthy food and is consumed in various forms such as whole tempeh and formulated tempeh, and separately as active compounds extracted from tempeh (Astuti *et al.* 2000, Nakajima *et al.* 2005). In Indonesia, tempeh is usually produced in traditional home industries. Yeast diversity in tempeh production has scarcely been studied, even though the occurrence of yeast in tempeh has been reported. Previous research mostly used conventional methodology that provided limited information on culturable yeasts. Furthermore, such methods are time consuming and require considerable effort (Pham *et al.* 2011 and often failed to detect the uncultivable microorganisms (Aslam *et al.* 2010).

On the basis of yeast diversity analyses through culturing, we recently reported that yeast were present at all stages of tempeh production in two home industries in Bogor (Efriwati *et al.* 2013). The yeast population was dynamic over time and differed depending on the method of tempeh production. We found approximately 88 yeast phylotypes associated with tempeh production. This number could be less than the actual yeast population since the majority of microorganisms, including yeast cannot be cultured

Both ITS-PCR RFLP and ITS-PCR T-RFLP profiles have been used as a technique for yeast community analyses (Pham *et al.* 2011, Hamby *et al.* 2012). A similar technique has been reported by Seumahu *et al.* (2012) to fingerprint a number of tempeh samples based on fungal community analysis. This study focused on yeasts and aimed to analyse the yeast diversity on tempeh produced by two different home production methods in Bogor. The study used ITS and 5.8S rRNA gene region analyses using T-RFLP on DNA directly extracted from samples of tempeh.

Materials & Methods

Sampling and samples for analysis

Sampling site and samples for analysis were similar to that of Efriwati *et al.* (2013), from two) tempeh home industries that have been explored previously by Barus *et al.* (2008). In the previous study, the samples were from fresh tempeh (Efriwati *et al.* 2013), while in this study, the samples had been frozen prior to analyses.

DNA isolation

Two grams of soybean or tempeh were taken from each samples for DNA isolation. Prior to isolation, all of the materials were pre-treated to eliminate the dietary components and other material according to Randazzo *et al.* (2002) with slight modification. In the preparation stage, soybean or tempeh were blended with a blender for 3 × 3 seconds on cold and aseptic conditions. The pellets were then crushed with pellet paste in liquid nitrogen. Metagenomic DNA was extracted by cetyl tri methyl ammonium bromide method (Sambrook & Russell 2000).

The resulting DNA were measured using a spectrophotometer and visualized by electrophoresis. DNA with the criteria of high concentration (250-350 ng/μl), and free from proteins and RNA were used as DNA template for next step of analysis.

PCR amplification

Amplification of complex ITS and 5.8S rRNA (ITS1- 5.8S rRNA- ITS2 gene) of yeast was done according to Carvaho *et al.* (2005) with modification. Modifications were in using Taq polymerase (Fermentas, USA) and optimization for yeast DNA amplification from tempeh. Each 50 μl of reaction mixture consisted of 0.46 μM FAM labeled ITS1 forward primer (5'-6-FAM-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 not labeled (5'-TCCTCCGCTTATTGATAGC-3'), 2,16 mM dNTP Mix, 2 mM MgCl₂, 1.6 units taq DNA polymerase, 1 × PCR buffer, and 0.5 μl (100-200ng) DNA template. Samples were amplified in PCR machine (Applied Biosystems Verity™. 96 well) with the temperature program consisted of an initial heat denaturation step of 94°C for 3 min and then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 2 min, followed by 10 min at 72°C. Ten micro liters of each PCR products was analyzed by electrophoresis in 1% gel agarose. Size of PCR product was estimated with Gel Doc™ EZ System (Bio-Rad) and O'range Ruler™ 50 pb DNA Ladder as a marker. PCR products were purified with Gene Jet™ PCR Purification Kit (Fermentas, USA) in accordance with the manufacturer's instructions.

Restriction of PCR products and T-RFLP

Purified PCR products were cut using restriction enzyme *Hae*III (Fermentas, USA). Reaction conditions were 15 unit restriction enzyme, 1x restriction buffer and 10 μl (100-200 ng) of

DNA in 20 µl total reaction. These mixtures were incubated and inactivated in accordance with the manufacturer's instructions. Restriction products were precipitated for drying which conducted based on standard procedure of Sambrook & Russell (2000). T-RFLP analysis was done by PT Wilmar Benih Indonesia, Bekasi, Indonesia.

Data processing

Diversity of yeasts were stated as number of phylotypes as labeled terminal fragment (TRF) with different length of fragments. TRF result of T-RFLP analysis was first normalized as suggested by Dunbar *et al.* (2001) and Blackwood *et al.* (2003). Only TRF which size is > 50 bp and height > 50 fluorescent unit (FU) is regarded as fragments that satisfy terms, less than that is considered as noise. Cumulative value of TRF sample must be greater than 10,000 FU.

Yeast phylotype richness (S) is the total of TRF peaks found in each restriction in each sample. TRF data from all stages of tempeh production of both methods were aligned using T-align program in <http://inismor.ucd.ie/~talign/>. (Smith *et al.* 2005) with a confidence interval of 0.5 bp. The results obtained in the form of a decimal fraction were rounded to the nearest number of TRF. The same TRF length represented one phylotype. Cluster analysis of all yeast phylotypes at all stages and two tempeh production methods was done using UPGMA analysis of http://insilico.ehu.es/dice_upgma/.

Results

Tempeh contains different sizes of amplicon of complex yeast ITS and 5.8S rDNA regions. One to three fragments (Fig. 1) were revealed at certain stage of tempeh production, but overall about seven fragments (from 326 bp to 720 bp) were detected from all stages of both tempeh production methods. Fragment size might be either varied or similar among stages and between tempeh production methods (Fig.1).

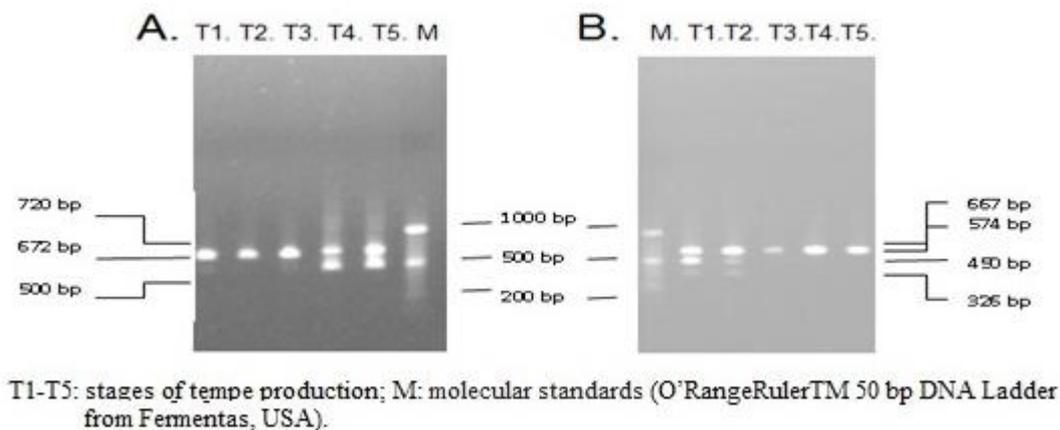


Fig. 1 – Amplicons of complex ITS and 5.8S rDNA region of yeast in tempeh production method A (A) and B (B).

The total number of yeast phylotypes during tempeh production in method A (5 – 17 phylotypes) was relatively similar with those in method B (6 -17 phylotypes) (Tabel 1 and Fig. 2). The number of yeast phylotypes in both tempeh production methods were similar at the starting point of making tempeh (T1) and then fluctuated at further stages (Fig. 2). These fluctuations were contributed by both some newly found and disappearing phylotypes (Tabel 1). The highest yeast phylotype occurred at different stages at two tempeh production methods. In method A, T3 showed the highest phylotypes. In contrast to method A, the highest phylotype occurred at T5 in method B. The major discrepancy between the yeast community of the two tempeh production method occurred at T3. In method B, T3 contained the smallest number of yeast phylotype.

Table 1 Yeast status at each stages of tempeh production

Phyloptype status	Total number of phylotypes in each stage of tempeh production									
	Method A					Method B				
	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
Found / detected at stage	12	12	17	14	5	12	10	6	10	17
Detected in the previous stage	0	6	12	17	14	0	12	10	6	10
Disappeared from previous stage	0	6	2	7	13	0	2	7	3	3
Newly found	0	6	2	2	0	0	2	2	6	10
Reappeared	0	0	5	2	4	0	0	1	1	0

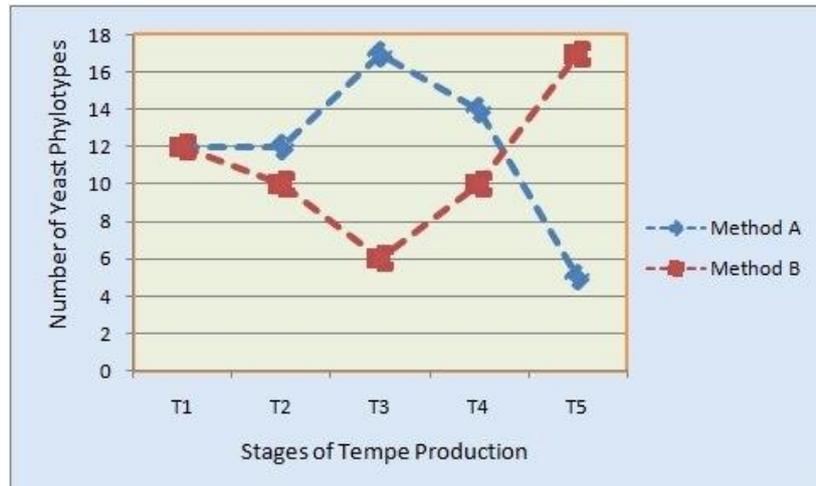


Fig. 2 – Yeast population dynamic at two different methods of tempeh production

Certain yeast phylotypes were common in both methods of tempeh production while others were specific to certain methods. The total yeast population in all stages and both method of tempeh production was 43 phylotypes. In method A there was 21 phylotype, whereas in method B was 30. Nine of 43 yeast phylotypes were common in both methods. Therefore, only 13 yeast phylotypes are specific to tempeh production method A and 21 were specific to method B (Fig. 3).

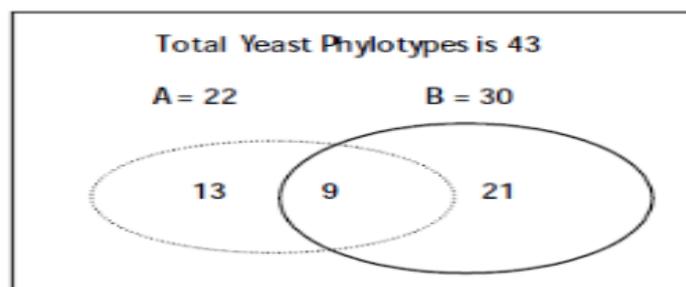


Fig.3 – Comparison of Total Yeast Phylotypes in Method A (A) and B (B)

On the bases of similarity index, yeast phylotypes during tempeh production of method A can be divided into two group communities. The first community composed of the phylotypes of T1, T2, T3 and T4. The second community composed of that of T5 phylotypes. This community was closer to T3 method B yeast community. The yeast communities in method B was also formed three clusters. Those of T4 and T5 formed a community that was nearly exclusively (> 75% of occurrences) different from the communities in other stages in method B or method A. However, T1 and T2 yeast community of method B was closely similar (Fig. 4).

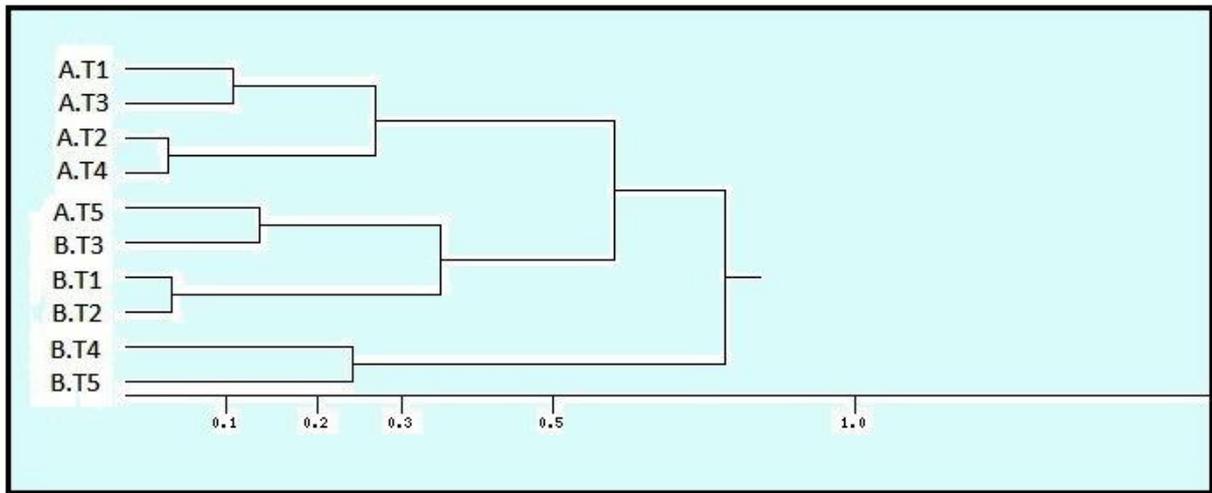


Fig. 4 – Yeast community clusters at various stages of tempeh production methods (A, B= method of tempeh production, T= stages in tempeh production)

During tempeh production, some phylotypes were detected only at certain stages, while the other phylotypes were present at several stages with fluctuating relative abundance from one stage to others (Fig. 5). Among nine of the common yeast phylotypes in both methods, two phylotypes, i.e. phylotypes with length of TRF 114 bp and 161 bp, were dominant during tempeh production. These phylotypes were present at three stages of method A and four stages of method B. These phylotypes were predominating and present with the relative abundance ranging from 0.9 % to 83% and 0.3 % to 42%, respectively.

Two (442 bp and 475 bp yeast phylotypes) out of 13 specific phylotypes in method A were present with high relative abundance (Fig. 5). These phylotypes were present at 4 of 5 stages of tempeh production with relative abundance ranging from 14.3 % to 31.2% and 1 % to 24.1%, respectively. On the other hand in method B, there was no specific phylotype like those at method A.

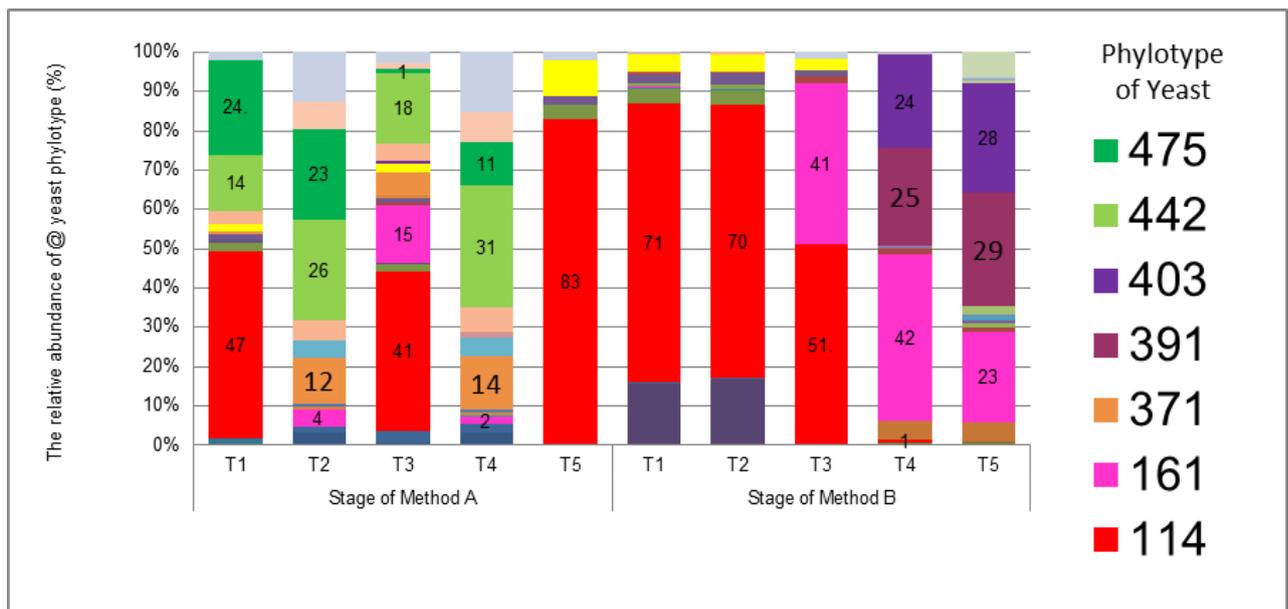


Fig. 5 – The relative abundance of yeast phylotypes from five stages of tempeh produced by method A and B

Discussion

Yeasts were part of microbial community in Indonesian tempeh (Ashenafi & Busse.1991; Efriwati *et al.* 2013). Ashenafi & Busse (1991) reported the existence of yeast in soaking soybean, but Efriwati *et al.* 2013 demonstrated that yeast were present throughout tempeh production. Ashenafi & Busse (1991), Efriwati *et al.* (2013) detected yeasts by plating method. This study, revealed the existence of yeast throughout tempeh production line using DNA directly extracted from samples followed by PCR amplification of complex ITS and 5.8S rRNA region.

The PCR products of complex ITS and 5.8S rRNA gene showed only seven fragments with size ranging from 326 bp to 720 bp (Fig. 1). One ITS fragment (326 bp) was apparently shorter than the size recognised by Zarzoso *et al.* (1999). The ITS fragment length found by Zarzoso *et al.* (1999) varied from 380 bp for *Yarrowia lipolytica* (CECT 1240) and *Pichia pastoris* (CECT 11078) to 1050 bp for the type strain of *Schizosaccharomyces pombe* var. *pombe*. This indicated that the shortest fragment found in this research might belong to a different or novel species.

In this study, the number of ITS-PCR fragments (Fig. 1) found were also less than the number of phylotypes (Table 1 and Fig. 2). This indicated that one fragment might represent more than one phylotype (TRF). Zarzoso *et al.* (1999) stated that in majority of cases, the PCR products from strains of the same species and from species of the same genus might have similar molecular sizes of ITS amplicons. They also stated that ITS-PCR RFLP patterns of 132 yeast species were frequently unique for species. However evaluation of ITS-PCR RFLP for differentiation and identification of brewing yeast and brewery 'wild' yeast contaminants showed that certain profiles were only genus specific, while other profiles were not specific to either species or genus (Pham *et al.* 2011). This indicates that the ITS-PCR T-RFLP is more sensitive than either ITS-PCR or ITS-PCR RFLP. T-RFLP process used automated detection equipment and had higher sensitivity to detect small changes in community profile between samples. T-RFLP possesses relative ease for quantitative analysis. It is not possible with other PCR non-automated methods (Spiegelman *et al.* 2005). Compared to other PCR-based genetic fingerprinting technique, T-RFLP has more advantages. T-RFLP is the fingerprinting technique for microbial community profiling (Juste *et al.* 2008) and Hamby *et al.* (2012) used ITS-PCR T-RFLP as an analytical tool for yeast community comparisons. This fingerprinting technique detects the diversity of yeast both qualitatively and quantitatively. Qualitatively, it was by the presence of a TRF (representing as phylotype) while quantitatively, it was based on the relative abundance of each phylotype shown as different area at each TRF.

Tempeh production methods affected yeasts diversity in terms of phylotypes number, specificity and relative abundance. The number and diversity of phylotypes was suppressed at T3 in the method that included the second cooking process (method B). About 7 yeast phylotypes of T2 disappeared after cooking process, indicating the lethal effect of cooking process to certain phylotypes. These phylotypes were considered as intolerant to heat from cooking process. Moreno *et al.* (2002), Nout & and had lethal effects.

Kiers (2005) also stated that cooking process reduced microbial populations in large numbers was lower than yeast diversity reported by Efriwati *et al.* (2013). In this study, the total of yeast phylotype found in all stages at both method of tempeh production was 43 phylotypes. This comprised of 22 phylotypes in tempeh method A and 30 phylotypes in tempeh method B, with 9 phylotypes as a common phylotypes (Fig. 3). Efriwati *et al.* (2013) reported about 88 phylotypes within similar samples, in which 26 phylotypes were in method A, 77 phylotypes were in method B and 15 phylotypes were common on both tempeh production method. Theoretically, analysis of yeast diversity after culturing process in selective media would detect only culturable phylotypes. Therefore, diversity of yeast phylotype of T-RFLP after plating would be less than direct T-RFLP from sample (without plating), because direct T-RFLP analysis from sample was able to detect all of yeast phylotype, both culturable and unculturable microorganism (Dicksved *et al.* 2007). Moreover, only few of microorganisms that can be cultured *in vitro* (Vartoukian *et al.* 2010). It was suspected that the majority of yeast phylotypes harboured in sample were in low abundance or the

population was under T-RFLP machine sensitivity (Phylotype value under qualification of FU value). In this case, isolating process might enrich lesser yeast population until greater than the FU value. Therefore, T-RFLP analysis after culturing would be suitable to determine species diversity, while T-RFLP from tempeh metagenome (direct DNA extraction) would be more appropriate to demonstrate species abundance.

In this study, two out of nine common strains (i.e. 114 bp and 161 bp phylotypes) were dominant with high relative abundance. These two phylotypes were present at 3 stages in method A and 4 stages in method B. The 114 bp phylotype was found more than 50% in some stage of tempeh production. At T5 stage in method A, this phylotype was 83% and in method B, at T1, T2 and T3 stage were 71%, 70% and 51%, respectively. The 114 bp phylotype was an interesting phylotype, because this phylotype was present in very high population in fresh tempeh in method A, but was not detected in fresh tempeh in method B. In method B, the relative abundance of this phylotype was very high at the beginning of soaking soybeans (T1) and decreases at the next stages. From T1 to T5, the relative abundance were 71% (T1), 70% (T2), 51% (T3), 1% (T4) and undetectable (T5). This phylotype is a common microbial in tempeh community and might indicate have a specific physiological function in tempeh.

Yeast population during tempeh production were dynamic over time and differed between the two methods of tempeh production. In this study, changes in microbial community structure at each stage and method were observed. T-RFLP method could monitor the changes of microbial community structure and composition due to different treatment and time (Jernberg *et al.* 2005, Dicksved *et al.* 2007) and provide a high compromise between information obtained and labor intensity (Schutte *et al.* 2008).

The advantages of direct T-RFLP analysis from sample is its ability to detect the structure and composition of microbial communities in actual condition, besides its ability to detect both culturable and unculturable microorganisms (Dicksved *et al.* 2007). This study concerned with both culturable and unculturable yeast. We described the culturable yeast from similar samples in a separate report (Efriwati *et al.* 2013). The unculturable diversity of yeast phylotype in this study (Fig. 6) was obtained by subtracting the whole diversity of yeast phylotypes detected in this study with the same phylotypes obtained in culturable analysis (Efriwati *et al.* 2013). The unculturable yeasts were present only in a small portion, and contrast to that of Vartoukian *et al.* (2010), Aslam *et al.* (2010). This might be due to the low abundance of dominant yeasts as discussed above, or the complexity of tempeh microbiota. Microbial communities in samples underwent direct T-RFLP analysis were not only yeast, but an extremely complex community which includes various kind of mold, lactic acid bacteria, gram positive and negative bacteria (Moreno *et al.* 2002, Barus *et al.* 2008, Nuraida *et al.* 2008, Seumahu *et al.* 2012, Efriwati *et al.* 2013). There is some evidence that T-RFLP could not effectively determine phylotype richness in extremely complex communities. Its applicability is more superior in simple communities (Dunbar *et al.* 2000). T-RFLP analysis might be more effective to determine phylotype diversity in yeast communities obtained after plating on selective media, because other microorganisms had been eliminated. This could be the reason that TRFLP analysis after plating generated more diverse yeast than T-RFLP analysis conducted directly from samples. In this study 43 yeast phylotypes were detected in T-RFLP directly from samples while there were 88 phylotypes for T-RFLP conducted after plating. When combined (culture dependent and culture independent method) could show 104 yeast phylotypes. The culture dependent method (T-RFLP analysis after plating) and culture independent method (T-RFLP analysis directly from samples) could complement each other to reveal yeast diversity in tempeh.

More robust analysis and database are essential, due to the fact that most phylotypes could not be identified referring with existing database. Meanwhile, there is high probability to find novel yeast species from this study. The high population of cultured yeast will provide more opportunities to identify individual colonies. Clone libraries technology can be used as an alternative way to obtain sequence information of TRF for further analysis (Juste *et al.* 2008).

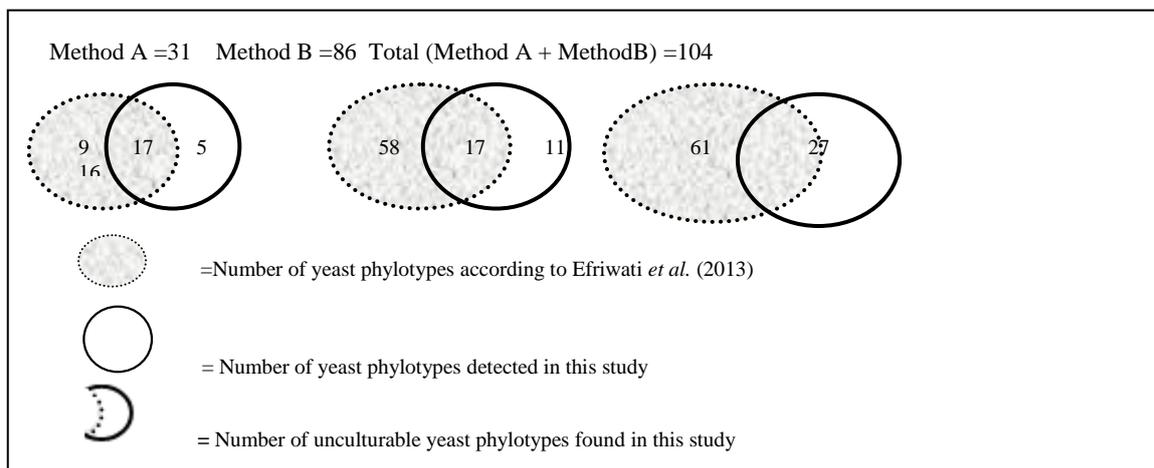


Fig. 6 – Comparison of number of yeast phylotypes detected in this study (by T-RFLP direct from samples) and Number of yeast phylotypes according to Efriwati *et al.* 2013 (T-RFLP after plating) from tempeh in method A, method B, and combined method (method A + method B).

In conclusion, employing metagenome analysis we could identify 43 yeast phylotypes in all stages of both tempeh production methods. All of yeast phylotypes could be grouped into five unique communities. There were two yeast communities in tempeh produced by method A, and three groups of yeast communities present in tempeh produced by method B. Nine phylotypes were found to be common and two of them were dominant in both tempeh production methods. Some phylotypes were specific in one of the stages or in tempeh production methods.

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