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Population Dynamics of Yeasts and Lactic Acid Bacteria (LAB) During Tempeh Production

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Yeasts and lactic acid bacteria (LAB) are commonly found in tempeh and has been studied separately. However, comprehensive study on population dynamics of yeasts and LAB during tempeh production, including the effect of the difference tempeh production methods has not been reported. This research was aimed in studying the effect of different methods of tempeh production applied in tempeh home industry on the dynamics of yeast and LAB communities. Population dynamics was expressed as both changes of colony number and its phylotype. Samples were obtained from five stages and from two different methods of tempeh production. Observations were carried out employing colony counting on selective media followed by Terminal Restriction Fragment Length Polymorphism (T-RFLP). The study indicated that the population of yeasts and LAB during tempeh production were dynamic and different between these methods. Tempeh production methods affected the presence of yeasts and LAB population as indicated by difference in colony number, the number and diversity of phylotype, as well as number of specific phylotypes grew on plates.

Keywords: tempeh, T-RFLP, yeast, lactic acid bacteria

INTRODUCTION

Tempeh production in Indonesian is mainly carried out by home industry that has no specific standard. Astuti *et al*. (2000) stated that tempeh production methods was different from one region and one producer to another. One of the significant different in tempeh production is whether or not it involved second cooking of soybean. This difference might cause a significant of variations in tempeh quality in terms of texture, taste, and aroma.

Tempeh production method without second cooking commonly performed by tempeh home industry in Bogor. Four out of five tempeh home industry studied by Barus *et al*. (2008) used no second cooking. Only one of tempeh home industry does the second cooking, namely WJB home industry that produce WJB tempeh. EMP tempeh produced by EMP home industry without second cooking had bacterial population that was higher than those of WJB tempeh (Barus *et al*. 2008). Further, they also stated that bitterness of EMP tempeh was also higher than that of WJB tempeh.

Yeasts and lactic acid bacteria (LAB) in tempeh have been reported by a number of researchers. Nout and Kiers (2005) stated that these two microbes were commonly found in tempeh. Yeast has not been studied comprehensively during soybean tempeh production, but Feng *et al*. (2007) report numerous yeast species grew in barley tempeh. LAB were found in the raw materials and during production of tempeh in home industry in Malaysia (Moreno *et al*. 2002), starting from raw materials, in the stage of soybean soaking, soybean boiling and in fresh tempeh. Until now the study of yeast and LAB in Indonesian tempeh is limited to the CFU value and listed several yeast and LAB. There is no comprehensive study on the population dynamic of yeasts and LAB during tempeh production, including the effect of different production methods on the presence of yeasts and LAB. Above all, this information is necessary for the development of quality traditional tempeh Indonesian. Nout and Kiers (2005) concluded that

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LAB at soaking and mold fermentation stage were important to reduce the level of pathogenic and spoilage bacteria in tempeh. The role of yeast during tempeh production is still unclear. Feng *et al.* (2005, 2007) showed that in barley tempeh, the inoculation of yeasts and LAB with *R. oligosporus* simultaneously in certain concentration does not inhibit the growth of *R. oligosporus*.

Studies on population dynamic of yeasts and LAB were limited to CFU value analysis. However, cultivation can underestimate the microbial diversity, as media may not be sufficiently selective and furthermore is laborious and time-consuming for monitoring population dynamics. This method also often failed to show the uncultivable microbes (Aslam *et al.* 2010). Molecular approach using Terminal Restriction Fragment Length Polymorphism (T-RFLP) could be expected to solve these problems. This method offers a compromise between the information gained and labor intensity (Schutte *et al.* 2008) and can be used to monitor the changes in the structure and composition of the microbial community due to a different treatment and time (Jernberg *et al.* 2005; Dicksved *et al.* 2007).

We aimed to study of yeast and LAB the effect of different production methods on population dynamics of yeasts and LAB during certain stages of tempeh production. The information obtained from this research is important to provide a basis to develop a defined starter culture for better quality of tempeh production.

MATERIALS AND METHODS

Sampling and Samples for Analysis. The study was conducted on samples taken from two tempeh home industries that have been explored previously by Barus *et al.* (2008) that were EMP and WJB tempeh home industry and further reference to method A and B, respectively. Preliminary research was done in advance to observe more thoroughly to ensure any difference between the two methods of tempeh production (Figure 1). The result of the observation was used as the bases for deciding the stages of tempeh production for sampling.

Samples were taken aseptically from each tempeh home industry location. Samples were taken in ice box for processing as soon as arriving at the laboratory (< 30 minutes). Samples were taken at five tempeh production stages, that were early stages (T1) and end (T2) of soaking soybeans and early stages (T3), middle (T4) and end (T5) of tempeh incubation (Figure 1). Sampling was done twice from two cycles of tempeh production with one day interval.

Enumeration and Microbiology Analysis. Microbiological analysis carried out by culturing, using modified MRS agar (Difco) supplemented with 0.2% sodium azide and yeast extract malt agar/YMA (Difco) containing 250 mg/liter chlortetracycline and 250 mg/liter chloramphenicol to enumerate the total LAB and yeasts following Plengvidhya *et al.* (2007). Ten grams of each sample was crushed and homogenized in 40 ml of sterile saline (0.85% NaCl). Then a serial dilution was made for each sample. From each dilution, a 100 µl aliquot of suspension plated on modified MRS agar and YMA to enumerate the total LAB and yeasts. Plating was done in two replicates and incubated is at 30 ^{o}C for seven days for LAB and at 25-27 ^{o}C for two days for yeasts (Plengvidhya *et al.* 2007). All of colonies on plate were counted manually. The total number of yeast and LAB colonies on plates was expressed as the number of colonies (cfu) per gram sample. Population dynamics of the colonies number were change of yeasts and LAB for tempeh production at every stage of tempeh production (stage T1, T2, T3, T4, and T5) in both tempeh production methods (Method A and B). Representative plates of all yeast and LAB colony presence both on each stage and method then were used as DNA source for phylotype analysis using T-RFLP molecular approach.

DNA Isolation of Total Genomic Yeast and LAB from Plate. The colonies of yeast and LAB from representative plates were harvested from each plate separately using cotton bud swap and then rinsed with sterile saline solution (0.85% NaCl). Microbial suspension obtained was centrifuged at 10,000 rpm for 10 minutes of at 4 ^{o}C. The pellets were crushed with pellet pestle in liquid nitrogen. Total DNA was extracted by cetyl tri methyl ammonium bromide method (CTAB) (Sambrook & Russell 2000).

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

PCR Amplification. Amplification of two ITS regions and 5.8S r RNA gene of yeast used Carvaho *et al.* (2005) method 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'-

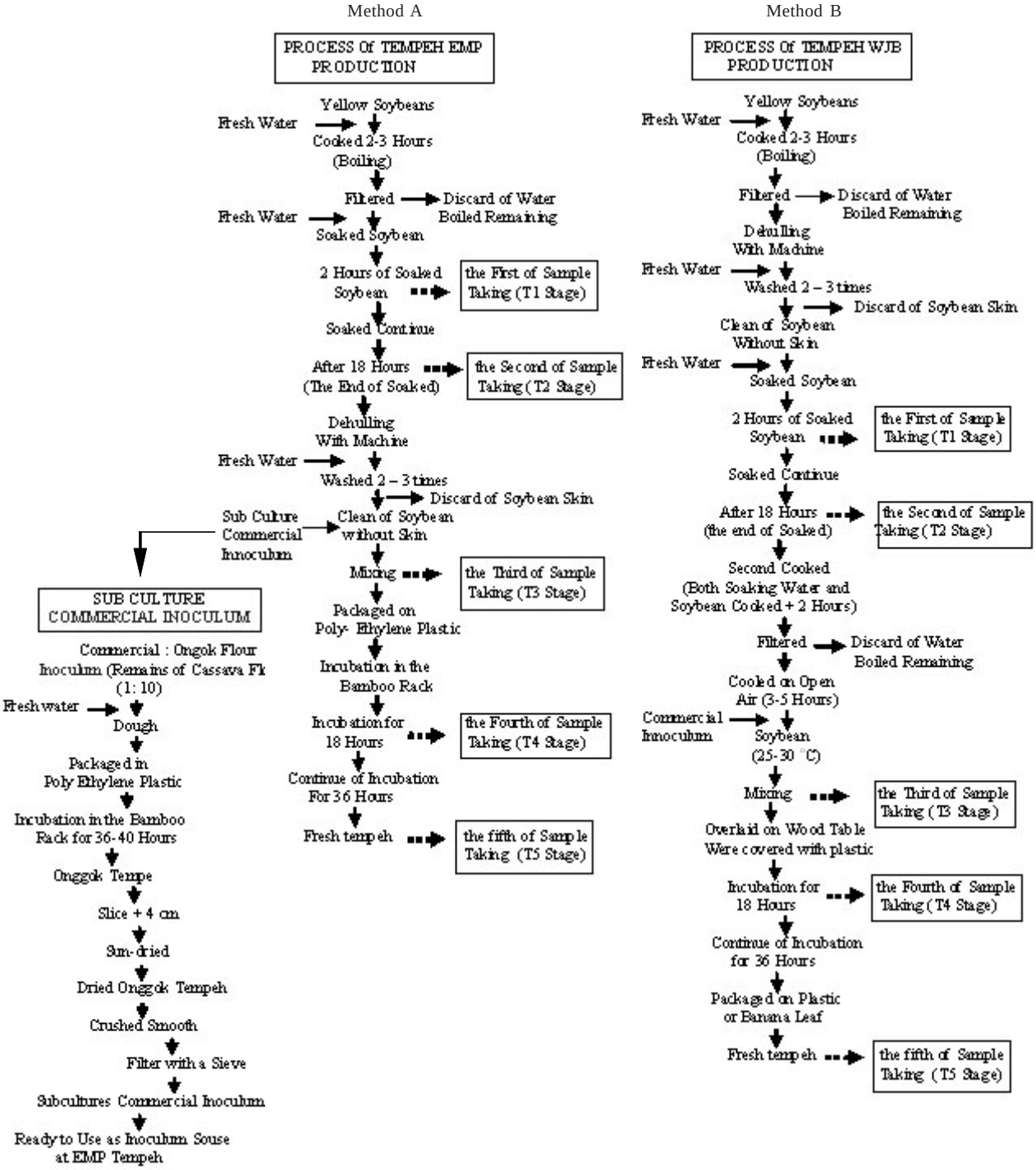

Figure1. Tempeh production line (method A and method B) in two home industries in Bogor and its sampling points.

6-FAM-TCCGTAGGTGAACCTGCGG-3‘) and reverse primer ITS4 not labeled (5’-TCCTCCGCT TATTGATAGC-3'), @ 2.16 mMdNTP Mix, 2 mM $MgCl_2$, 1.6 units taq DNA polymerase, 1 × PCR buffer, and 0.5 µl (100-200 ng) DNA template. Samples were amplified in PCR machine (Applied Bio systems 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 0.8% gel agarose. PCR products were purified with Gene Jet ™. PCR Purification Kit (Fermentas, USA) in accordance with the manufacturer’s instructions.

The specific primers (reverse SG-Lab-0677) (Jernberg *et al.* 2005; Dicksved *et al.* 2007) were used to amplify 16S rRNA regions of LAB. Modifications were in using Taq polymerase

(Fermentas, USA) and optimization for LAB DNA amplification from tempeh. Each 50 µl of reaction mixture consisted of 0.74 µM @ 7f forward primer labeled with FAM (5'-6-FAM-AGAGTTTGATC/TA/CTGGCTCAG-3') and unlabeled reverse primer SG-Lab-0677 (5'-CACCGCTACACATGGAG-3'), 2.5 mm @ d-NTP Mix, 3 mM $MgCl_2$, 1.5 units taq DNA polymerase, 1 × PCR buffer, and 0.5 µl (100-200 ng) DNA template. Samples were amplified in PCR machine (Applied Bio systems Verity™96 well) with the temperature program consisted of an initial heat denaturation step of 95 °C for 5 min and then 30 cycles of denaturation at 95 °C for 40 seconds, annealing at 55 °C for 40 seconds, and extension at 72 °C for 1 min, followed by 7 min at 72 °C. Ten micro liters of each PCR products was analyzed by electrophoresis in 0.8% gel agarose. PCR products were purified with Gene Jet™ PCR Purification Kit (Fermentas, USA) according to the procedure.

Restriction of PCR Products. Purified PCR products were cut using restriction enzyme *Hae*III (Fermentas, USA) for yeasts and *Msp*I restriction enzymes (Fermentas, USA) for LAB. Reaction conditions were 15 unit restriction enzyme, 1 x restriction buffer and 10 µl (100-200 ng) of DNA in 20 µl total reaction. Incubated and inactivated in accordance with the manufacturer's instructions. Restriction products were precipitated for drying conducted based on standard procedure (Sambrook & Russell 2000). T-RFLP analysis done by sent the restriction products of samples to the Laboratory of Biotechnology Department PT. Wilmar Seeds Indonesia located on JABABEKA Bekasi West Java, Indonesia.

Data Processing of T-RFLP. Diversity of yeasts and LAB were stated as number of phylotype as labeled terminal fragment (TRF) with different length of fragments. Previous TRF result of T-RFLP analysis has been normalized as such Dunbar *et al.* (2001) and Blackwood *et al.* (2003) procedures. Only TRF which size is > 50 bp and high > 50 fluorescent unit (TU) is regarded as fragments that satisfy terms, less than that is considered as noise. Cumulative value of TRF sample must be > 10,000 FU.

TRF Yeast/LAB phylotypes 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 progran 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. Population dynamics in term of phylotypes were change of diversity and number of phylotype of yeast and LAB at every stage of tempeh production (stage T1, T2, T3, T4, and T5) in both tempeh production methods.

RESULTS

Population Dynamics of Yeast and LAB as Colonies Number on Plate. The number of yeast and LAB colonies that grew on the plates during tempeh production was fluctuated from one to other stages depending on tempeh production methods (Figure 2). At T1, the number of yeast and LAB colonies in methods A is lower than those of method B. From T1 to T2, the population of those microbes

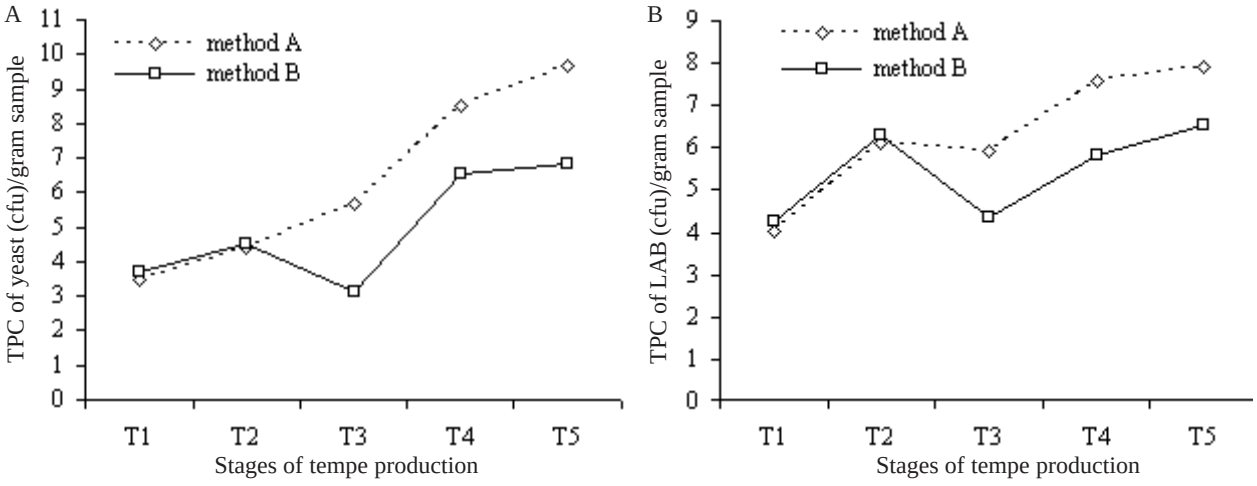

Figure 2. Dynamics of yeast (A) and LAB (B) colonies number at 5 stages (T1, T2, T3, T4, and T5) of tempeh production in method A and method B.

increased in relatively similar number on both methods. The yeast population increased approximately 1 log cfu/g sample and LAB population increased approximately 2 log cfu/g samples.

From T2 to T3, the number of yeast and LAB colonies decreased in method B. This was contrast to method A in which those microbial population was not decreased. In method A, the number of yeast colonies increased from T2 to T3, while that of LAB was relative constant.

Starting at T3, the yeast population was different between the two methods more than 2 log cfu/g sample and close to 2 log cfu/g samples for LAB population. However, from T3 to T5 they increased in relatively similar number such that at incubation stage (T3, T4, and T5) the population of yeast and LAB was higher in method A than that of in method B.

Population Dynamics of Yeast and LAB in term of Phylotypes. The population of yeast and LAB phylotypes that grew on the plates in both tempeh production methods ware fluctuated from one stage to other stages (Figure 3). In method A, the number of yeasts phylotypes was 8 to 11 phylotypes, whereas in method B ranged from 9 to 55 phylotypes. The highest number of yeast phylotypes was found in method A at T2 stage i.e. 55 phylotype. Unlike yeast phylotypes, the highest number of LAB phylotypes was found in method B at T3 (54 phylotypes). The number of LAB phylotype in method B ranged between 6 and 54 phylotypes, whereas in method A ranged from 10 to 12 phylotypes.

The actual phylotype present in a particular stage is the sum of the phylotypes presence in the previous stage - the number of phylotypes disappeared from previous stage + the number of newly found phylotypes + the number of reappeared phylotypes. At T2 in Method A, 8 yeast phylotypes was found at T1. From T1 to T2, two phylotypes disappeared and 5 phylotypes were newly detected. At this period, there was no reappeared phylotypes. Therefore, at T2 the population became 11 phylotypes (Table 1). The number of phylotypes reappeared were only found at some stages (T3, T4, and T5), while no in T1 and T2. This phenomenon was also occurs on the population dynamics of yeast in method B and LAB phylotypes in both methods (Table 1 & 2).

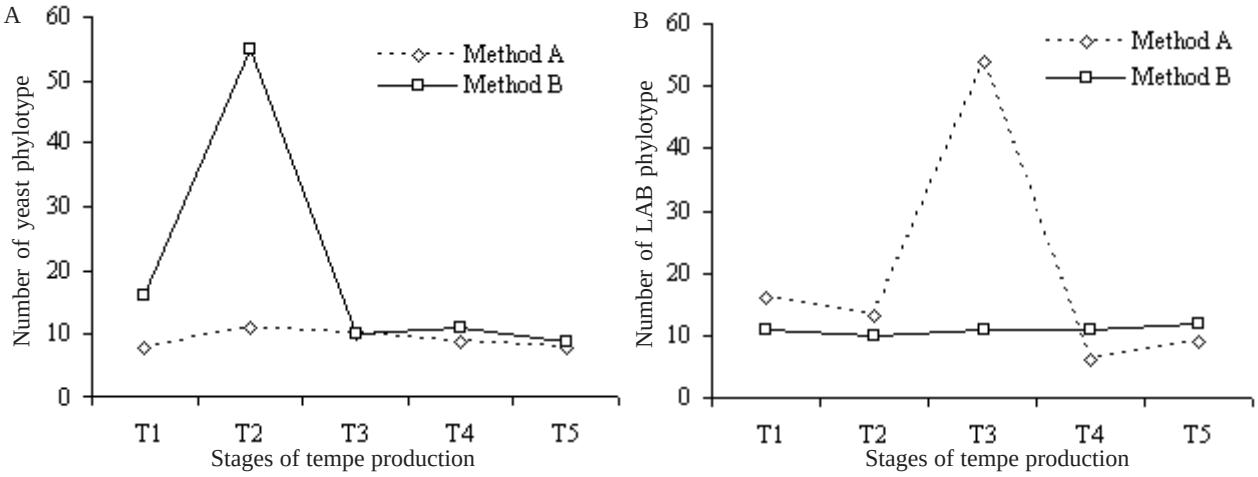

Figure 3. Dynamics of yeast (A) and LAB (B) phylotypes at 5 stages (T1, T2, T3, T4, and T5) of tempeh production in method A and method B.

Table 1. Yeast phylotype dynamics during tempeh production in method A and method B

Phylotype status	Total number of phylotypes in various stages									
	Method A					Method B				
	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
Found/detected at stage	8	11	10	9	8	16	55	10	11	9
Detected in the previous stage	-	8	11	10	9	-	16	55	10	11
Disappeared from previous stage	-	2	9	6	2	-	7	50	9	6
Newly found	-	5	8	5	0	-	46	5	8	2
Reappeared	-	-	0	0	1	-	-	0	2	2

Table 2. Lactic acid bacteria (LAB) phylotype dynamics during tempeh production in method A and method B

Phylotype status	Total number of phylotypes in various stages									
	Method A					Method B				
	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
Found/detected at stage	16	13	54	6	9	11	10	11	11	12
Detected in the previous stage	-	16	13	54	6	-	11	10	11	11
Disappeared from previous stage	-	6	4	51	4	-	4	8	5	4
Newly found	-	3	43	3	4	-	3	9	0	2
Reappeared	-	-	2	0	3	-	-	0	5	3

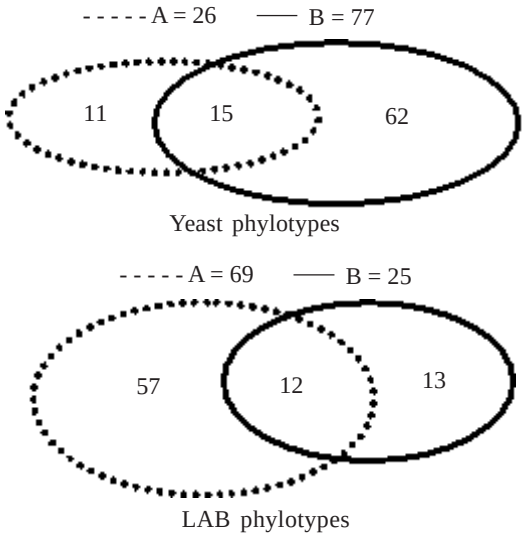

Figure 4. Comparison of total yeast and LAB phylotypes in method A (A) and method B (B). Total yeast phylotypes is 88, total LAB phylotypes is 82.

Certain yeast and LAB phylotypes is common in both methods of tempeh production while other its is specific to certain methods. Total yeast phylotype in all stages of method A was 26, whereas in method B was 77. Fifteen of yeast phylotypes is occurred in both methods. Therefore, only 11 of yeast phylotypes are specific to method A of tempeh production and 62 were specific to method B (Figure 4). A similar pattern was also found for LAB. About 57 out of 69 LAB phylotypes on method A was specific to methods A of tempeh production and 13 out of 25 LAB phylotypes were specific to method B. About 12 LAB phylotypes were commonly found in both methods of tempeh production (Figure 4).

DISCUSSION

This study showed that yeast and LAB were a common microbial community during tempeh production. The population of yeasts and LAB for tempeh production were dynamic over time and differed between the two methods of tempeh production. Regardless the method of tempeh production, the occurrence of yeast during tempeh production was reported by Ashenafi and Busse (1991); Feng *et al*. (2007). However the studied the not on the yeast dynamic during tempeh production. Yeast population were detected in water of soaking soybean nearly reach 10^6 cfu/ml in acidified water and 10^8 cfu/ml in unacidified water (Ashenafi & Busse 1991) and numerous yeast species grew in barlay tempeh (Feng *et al*. 2007). This study found the yeast population was maximum at final product and amounted to 9.70 log cfu/g in tempeh production method A and 6.85 log cfu/g in method B. Previous research also showed the occurrence of LAB during tempeh production (Mulyowidarso *et al*. 1990; Ashenafi & Busse 1991; Moreno *et al*. 2002). This study showed the LAB population was maximum at final product and amounted 7.91 log cfu/g in tempeh production method A and 6.54 log cfu/g in tempeh production method B. Mulyowidarso *et al*. (1990) found as much as 6-7 log cfu/g population of individual *Lactobacillus casei* and *Streptoccocs fuecium* at 36 hours incubation which prepared at laboratory with the method similar to tempeh production method B. Meanwhile Moreno *et al*. (2002) reported the occurrence of LAB in tempeh in the range of 6.8-9.9 log cfu/g.

Overall in this study, during the production of tempeh (from T1 to T5), the population of yeast and LAB in terms of cfu value in method A (without second cooking) were very different than those of method B. During tempeh production with method A, the population of yeast increased from T1 to T5. Whilst the LAB population increased from T1 to T2, then remained relatively constant from T2 to T3 and increased again from T3 to T5. In method B (with second cooking), yeast and LAB population increased from T1 to T2, then their population decreased from T2 to T3, but increased again from T3 to T5. This difference was due to the decreasing population from T2 (late stage of soaking the soybeans) to T3 (early stage of tempeh incubation) of method B. In method A without second cooking, their population did not reduce. Thus, the cooking process after the end of

soaking soybeans affected on both yeast and LAB population in particular reducing the population at T3. Moreno *et al*. (2002) stated that soybean cooking process reduced microbial populations in large numbers and further Nout and Kiers (2005) that the cooking process had lethal effects. The second cooking process will affect microbial population in next stage. At stage T3 until stageT5 (the initial incubation until the end of the incubation of tempeh / when fresh tempeh produced), the yeast population in method A was almost 3 log cfu/g sample higher than those of method B and for LAB method A higher than method B was almost 2 log cfu/g sample. Tempeh production methods affected the presence of yeasts and LAB in terms of colonies number.

Tempeh production methods also affected the presence of yeasts and LAB in terms of phylotype number and phylotype diversity. The lethal effect and suppression of microbial populations due to cooking process (Moreno *et al*. 2002; Nout & Kiers 2005) also showed in terms of phylotypes number (Figure 3) and phylotypes diversity (Tables 1 & 2). The number of yeasts phylotype method B of tempeh production was reduced dramatically from 55 phylotypes at T2 to only 10 phylotypes at T3. The cooking process after T2 caused most of the 55 yeast phylotypes undetectable at T3 (Table 1). There was a possibility that these phylotypes were un tolerant to heat from cooking process. Meanwhile, the LAB phylotypes slightly increased from 10 at T2 to 11 at T3 (Figure 2) in method B of tempeh production. However, these 11 LAB phylotypes were actually the result of 8 LAB phylotypes lost those had been detected in the previous stage and the addition 9 newly found LAB phylotypes (Table 2). The cooking process after T2 also caused most of LAB phylotypes (8 out of 10 LAB phylotypes) were undetectable in T3, leaved 2 LAB phylotypes detectable. In addition, comparing to method A (without second cooking), there was increasing LAB phylotype number from 13 at T2 to 54 phylotypes at T3. It was likely the number of LAB phylotypes in method B reduced by more than 8 phylotype. The presence of bacteria during tempeh incubation (fungal fermented) was associated with the presence of bacteria in the soaking of soybeans and bacterial survival ability at the end of cooking process after soybeans soaking (Mulyowidarso *et al*. 1990).

Yeasts and LAB population was also found at T3 in method B (Figure 1) after second cooking process for $\pm$ 2 hours. At this stage, the number of yeast and LAB in term of cfu was still found in high number, i.e. 3.11 and 4.36 log cfu/g samples respectively. Further, those of phylotypes were 10 and 11 phylotypes respectively (Figures 2 & 3; Tables 1 & 2). Moreno *et al*. (2002) stated that yeasts, mold and LAB could be found in the boiled soybean less than 4 hours.

It was difficult to ensure that all yeast and LAB population found was the population that resistance to the second cooking process. Before sampling at T3 stage, inoculum was added. Therefore, there was a possibility that some population of yeasts and LAB found at T3 were derived from the inoculum. No information of microbial in habitat of laru, yet (source of fungal inoculum). In addition, the time span between the time after cooking and the application of inoculum and also the interactions of workers, equipment, and the environment when fungal inoculum was given and soybean cooling may contribute to the re-association of microbes from environment to tempeh production systems, including yeast and LAB found at T3 in method B. It was stated that microbes found in traditional tempeh caused by ecological factors (Nout & Kiers 2005), the ability of microbes components grew in the mold fermentation conditions and their resistance after cooking process in the final soaking, and additional contamination after boiling (Mulyowidarso *et al*. 1990).

There was no correlation between yeast and LAB colonies number (Figure 2) and their phylotype number (Figure 3). In tempeh produced by the method A and method B, the number of yeast and LAB colonies at stage T5 was the highest, but the highest number of yeasts and LAB phylotype was not at stage T5. At stage T5, these yeast and LAB phylotypes were dominated by a few phylotypes. These phylotypes found at T5 were phylotypes that could survive from various previous treatments, including their ability to grow together with mold dominated by *Rhizopus oligosporus*, their biochemical ability to use soybean component from catabolic products or mold metabolism products, including the production of anti-microbe (Mulyowidarso *et al*. 1990; Feng *et al*. 2005, 2007).

Tempeh production methods affect phylotype specific presence of yeasts and LAB. Some yeasts and LAB phylotypes were found to be specific to a particular tempeh production methods, because it was only found in certain tempeh production methods and never found in other tempeh production methods. This specific phylotypes also differed between the two methods of tempeh production. Eleven yeast phylotypes found specific in method A, while 62 other yeasts phylotypes found specific in method B. A total of 57 LAB phylotype found specific in methods A and 13 other LAB phylotypes found specific in method B (Figure 4). Overall, yeast phylotypes found

in tempeh production with method B (77 phylotypes) were higher than method A (26 phylotypes). The number of LAB phylotypes found in tempeh production with method A (69 phylotypes) was higher than method B (25 phylotypes). If these findings were related to the methods and processes of tempeh production (Figure 1), it was found that the yeasts and LAB population were different due to different treatment and times. The T-RFLP molecular approach after plating could detect all of these, because the T-RFLP method could monitor the changes in microbial community structure and composition due to different treatment and time (Jernberg *et al*. 2005; Dicksved *et al*. 2007) and shown to provide a high compromise between information (Schutte *et al*. 2008).

The T-RFLP molecular approach after plating, although capable of detecting a total of 88 yeast and 82 LAB phylotypes, all of the results could not describe the whole of yeast and LAB phylotypes during tempeh production processes. The results of this study limited to yeast and LAB phylotypes found in the plate and were able to grow (culturable) on selective media. It was known that only a few of microorganisms that were easily cultured *in vitro* (Vartoukian *et al*. 2010). The majority of bacteria cannot be cultured (*unculturable*), and this may also happen to the yeast and LAB phylotypes in this study. It is important to proceed to the analysis using culture-independent method to reveal the entire diversity of yeasts and LAB phylotype during tempeh production. Moreover identification of the phylotypes has not been done. Identification of the phylotypes is needed to verify and validate the data refering on existing database.

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