Biodiversity of prokaryotic microorganisms in whey tofu from Lembang, Sumedang and Garut West Java

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Abstract. Tofu is one of the products processed from soybeans that contain nutrients for the body. Tofu from Lembang, Tofu from Sumedang and Tofu from Garut are three types of tofu that are produced in different places. Types of microorganisms found in whey can influence the flavor of tofu. This research was conducted to find out the type of microorganisms contained in whey tofu from Lembang, Sumedang and Garut through metagenome approach. Through this approach, microbial species in an environment can be known, even without the need to cultivate the microorganism. Whey samples filtered by cellulose acetate membrane of 0.2 µm. Total DNA was isolated by the Klijn method, and used as a template to obtain a fragment of 16S rRNA with ± 400 bp long using touchdown PCR. Amplicons obtained then separated again by DGGE analysis. Optimization of DGGE was made with 8% gel and a denaturant range of 30%-80%. The microorganisms contained in whey tofu from Lembang have closer homology to the Bacterium EM, microorganisms contained in Tofu from Sumedang were closer with Acinobacter sp. and Uncultured Bacterium, while microorganisms contained in Tofu from Garut were closer to Uncultured bacterium.

1. Introduction
Tofu is one of the processed products from soybeans that have good nutrition for the body. Tofu from Lembang, Tofu from Sumedang and Tofu from Garut are three types of tofu that are produced in different places but still close together (in one province, namely West Java). Although made from the same basic ingredients like soy, every tofu industry will produce different tofu products, both in terms of taste, aroma, texture, and suppleness. This difference can be caused by differences in treatment during the process of making tofu. The different types of coagulants used can also be a factor causing differences in each tofu product. Whey Tofu, which has been known as a by-product of tofu, in fact it can function as a coagulant in the process of making the next tofu. The type of microorganisms found in whey tofu is estimated to affect the flavor of tofu. Then a study was conducted to find out the types of microorganisms contained in the Lembang Tofu whey, Sumedang and Garut through the metagenome approach. Through this approach, microbial species in an environment can be known, even without the need for cultivation first.
2. Methods

2.1. Isolation of microorganisms by filtration method
About 100 mL of whey samples (Tahu Sumedang, Tahu Lembang and Tahu Garut Tofu were treated the same) were filtered with a 0.22 µm membrane filter, after previously being incubated at room temperature overnight. Screening is carried out in an aseptic environment. Microorganisms along with other deposits filtered in the filter were rinsed with STE buffer (10mM Tris-HCl pH 8.0; 0.1 M NaCl; 1 mM EDTA). The obtained suspension was put into 2 microcentrifuge tubes and centrifuged at a speed of 3000 / g. The supernatant is removed and the pellet is again rinsed with STE buffer at least 4 times. The rinsed pellets are then stored in a -20°C freezer until total DNA is isolated.

2.2. DNA isolation
Total DNA was isolated using an enzymatic-based lysis method which is a modification of the method proposed by Klijn et al. [1]. The enzymatic method was carried out as follows: cell pellets were suspended in 200 µL 10 mM Tris-HCl buffer (pH 8.0) containing 8 mg / mL lysozyme and incubated at 37°C for 1 hour. The cell was then lysed with the addition of 200 µL of lysis buffer containing 2% SDS, 0.8 mg / mL proteinase K, and 200 mM EDTA pH 8.0. The lysis process is carried out at 50°C for 30 minutes. Subsequently added 150 µL of cold C solution (60 mL CH3COOK 5 M; 11.5 mL glacial acetic acid; 28.65 mL H2O) and vortex for 10 seconds.

2.3. Amplification of 16S rRNA fragments
Amplification of the 16S rRNA fragment is a PCR touchdown amplification method performed by Ferris et al. [2]. The DNA polymerase enzyme used for PCR is the Taq DNA polymerase enzyme. A total of 30 µL PCR reaction mixture (Taq buffer PCR with (NH4) 2SO4 10x; 30 pmol primary P1 (forward); 30pmol primary P2-GC (reverse); 0.2 mM dNTPs; MgCl2 1.5 mM; 1 Taq enzyme unit DNA Polymerase; 0.5-2 µL DNA template) used for DNA chromosome amplification. The PCR cycle is 1 minute for denaturation at 94°C, 1 minute for annealing, and 1 minute for primary elongation at 72oC. In the first 10 cycles, the annealing temperature decreased regularly from 53°C to 43°C at intervals of 1°C per cycle. For the next cycle, the annealing temperature is carried out at 43oC. The last primer is extended for 7 minutes.

2.4. DGGE (Denaturant Gradient Gel Electrophoresis)
The separation technique by DGGE (Denaturing Gradient Gel Electrophoresis) is based on the Fischer and Lerman method [3]. There are several stages in conducting the electrophoresis, including preparation of reagents, making gels, preparation of samples, and electrophoresis. All equipment in electrophoresis must be clean and fat-free. For this purpose, the glass plates used as molds in the manufacture of gel are washed first with a detergent until clean, then dried and finally cleaned with 70% technical alcohol.

2.5. Phylogenetic analysis
In this research some data needs to be analyzed with the help of a computer program, including aligning the DNA sequence of samples with DNA sequences in GenBank, taking DNA sequences from GenBank, editing DNA sequences, aligning DNA sequences using ClustalW version 1.83, and tree construction phylogenetic using the Philip 3.62 program.

3. Results and discussion

3.1. Total DNA isolation
Until now, there is no universal method that can be used to extract total DNA from various other samples [4]. In this study, total DNA isolation was carried out using the enzymatic method Klijn et al., that had
been modified [1]. The enzymatic method was chosen based on the results of previous studies which stated that chromosomal DNA isolated using this method provided better purity than the physical destruction method. This method is modified so that the proteins that are isolated can be separated and do not interfere with the amplification process at a later stage. However, the disadvantage of this method is that there is less quantity of DNA obtained. This is due to the selectivity of enzymes that cause higher lysis selectivity [4].

3.2. Amplification of 16S RNA fragments
To identify microorganisms from whey samples, an approach based on the 16S rRNA gene sequence was performed using the DGGE fingerprinting technique. When using DGGE analysis, DNA fragments of about 200-500pb are needed to provide good analysis results Fisher and Lerman [3]. Before being used to amplify fragments of 16S rRNA genes of 200-500pb in size, first the amplification of 16S rRNA intact genes was carried out using universal primers namely forward (Bact27) and reverse (uni492R). This was done to ensure that the 16S rRNA gene from the whey tofu sample could be amplified. Next, touchdown PCR was amplified with P1-GC as forward and P2 as the reverse. The fragment was obtained by amplification of a portion of the 16S rRNA gene from the total DNA of Sumedang, Lembang and Garut Tofu samples.

The primary pair of P1-P2 can amplify bacterial groups and some archaeological groups [2] P1 is designed based on the sustainable sequence (conserve) of members of the bacterial domain (Escherichia coli positions 1392-1406) incorporated by 40 bases of GC-clamp. Theoretically, this primer amplifies 323 bp of the 16S rRNA gene. The amplification process with the primary pair is done with a touchdown system. The touchdown amplification system is intended to reduce primary specificity to increase diversity, where DNA templates with relatively low primary recognition can be amplified.

3.3. DGGE
The results of DGGE analysis as shown in Figure 1 show that the separation of the bands contained in the gel resulting from the silver nitrate staining, there are 6 bands for Lembang Tofu, 14 bands for Sumedang Tofu and 11 bands for Tofu Garut. By looking at the profile of the tape produced by each sample whey Tofu indicates that the three tofu whey contain different types of microorganisms.

![Figure 1. DGGE profile. (A) DGGE gel image, (B) Illustration image of DGGE profile. Position of wells in a row: 1 & 2 samples of Lembang; 3 & 4 Sumedang samples; 5 & 6 samples of Garut.](image)

The next step is to determine the nucleotide sequence of each band in the DGGE electropherogram. For this reason, it is necessary to isolate each DNA band. The tapes in the DGGE gel were cut and isolated using TE 1x buffer and heated for 2 hours at 50oC and incubated overnight at 37oC. The DGGE tape
that has been isolated has a low concentration and still contains GC-clamp, so before sequencing it is necessary to do the reamplification stage.

The 16S rRNA reamplification stage was carried out using P1 and P2 primers which were primers in the same order at the previous 16S rRNA amplification stage but in the P1 (forward) section it was designed not to contain GC-clamp. Results of the reamplification of around 400pb can be seen in Figure 2-4.

**Figure 2.** Electrophoresis results reamplification of tofu Lembang samples. (1) Lembang tape 1, (2) Lembang tape 2, (3) Lembang tape 3, (4) Lembang Ribbon 4, (5) Lembang Ribbon 5.

**Figure 3.** Electrophoresis results for re-amplification of the Sumedang Tahu sample. (1) Ribbon 1, (2) Ribbon 2, (3) Ribbon 3, (4) Ribbon 4, (5) Ribbon 5, (6) Ribbon 7, (7) Ribbon 7, (8) Ribbon 8, (9) Ribbon 9, (10) Ribbon 10, (11) Ribbon 11, (12) Ribbon 12, (13) Ribbon 13, (14) Ribbon 14.

**Figure 4.** Electrophoresis results for reamplification of Garut tofu samples. (1) Ribbon 1, (2) Ribbon 2, (3) Ribbon 3, (4) Ribbon 4, (5) Ribbon 5, (6) Ribbon 7, (7) Ribbon 7, (8) Ribbon 8, (9) Ribbon 9, (10) Ribbon 10, (11) Ribbon 11.
After re-amplification of the DGGE bands, an analysis is carried out by determining the nucleotide sequence through sequencing analysis. Sequencing analysis was carried out on several reamplified bands, by selecting bands that were in a different migration position from the results of the DGGE gel (Figure 1).

Analysis of each DNA sequence sequencing from 20 bands began with a comparative study with the 16S rRNA sequence in the GenBank data collection using the BLAST N program at the National Center of Biotechnological Information (NCBI) website. Data sets from the results of a comparative study are downloaded in the form of FASTA and then used in the alignment analysis. The alignment results are then used to construct phylogenetic trees.

3.4. Phylogenetic analysis
A more detailed description of the diversity of microorganism communities in Tahu Lembang, Tahu Sumedang and Tahu Garut was studied through phylogenetic analysis of DGGE bands. In this case diversity is seen based on the grouping of bacteria represented by each DGGE band. The nucleotide sequence of 16S rRNA gene fragments from each band can provide information at phylum, class and genus level. The size of gene fragments is unable to provide information down to the species level [5].

After a phylogenetic analysis of each whey tofu sample, a combined phylogenetic analysis of the three tofu whey samples was carried out, both for the Lembang Tofu whey, the Sumedang Tofu whey, and the Garut Tofu whey to see how the kinship of microorganisms in the three tofu whey. The results of the combined phylogenetic analysis of the tofu whey sample are shown in Figure 5.

Figure 5 shows that all isolates originating from Tahu Lembang whey form separate branches of a set of Tahu Garut whey isolates and Tahu Sumedang whey isolates have a fairly close kinship. The different types of microorganisms are likely to affect the characteristics of the tofu produced. To find out the relationship between types of microorganisms and the characteristics of tofu produced, an analysis of the aroma compounds present in each tofu and organoleptic tests were carried out.

Figure 5. Combined phylogenetic trees of the three whey tofu samples.

4. Conclusion
16S rRNA fragments from microorganisms in Lembang, Sumedang and Garut Tahu whey have been amplified with a size of around 400pb. Separation results through DGGE showed that there were 5 Lembang sample bands, 14 Sumedang sample bands and 11 Garut sample bands on electropherograms with different intensities. The results of homology analysis of the nucleotide sequences of each band showed that the microorganisms contained in Tofu Lembang, Sumedang and Garut were different, i.e. the microorganisms contained in Tofu Lembang whey had a closer kinship with Bacterium EM, the microorganisms contained in Tofu Sumedang had more kinship close to Acinetobacter sp. and Uncultured Bacterium, while microorganisms contained in Tofu Garut have a kinship closer to Uncultured Bacterium. The same results were also shown by phylogenetic analysis. The results of the phylogenetic analysis showed the presence of microorganisms in different tofu whey in separate
branches in the phylogenetic tree. This shows that the microorganism might be a new strain. The results of the phylogenetic homology analysis also showed that the three tofu whey contained different types of microorganisms where the microorganisms in the Sumedang and Garut Tahu whey had a fairly close kinship.

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References
[1] Klijn N, Weerkamp A H and de Vos W M 1991 Identification of Mesophilic Lactic Acid Bacteria by Using Polymerase Chain Reaction Amplified Variable Regions of 16S rRNA and Specific DNA Probes Applied and Environmental Microbiology 69 3607-3616
[2] Ferris M J, Muyzer G and Ward D M 1996 Denaturing Gradient Gel Electrophoresis Profiles of 16S rRNA Defined Populations Inhabiting a Hot Spring Microbial Mat Community Applied and Environmental Microbiology 63 340-346
[3] Fischer S G and Lerman L S 1983 DNA fragments differing by single base pair substitutions are separated in denaturing gradient gels, Correspondence with melting theory Proceeding of the National Academy of Sciences, U.S.A 80 1579-1583
[4] Yohandini H 2008 Cell lysis variation on assessment of microbial diversity based on ribotyping analysis (Bandung: Institut Teknologi Bandung)
[5] Muyzer G and Smalla K 1999 DGGE/TGGE a method for identifying genes from natural ecosystems Current Opinion Microbiology 2 317-322