PCR APPROACH FOR RAPID DETECTION OF *Escherichia coli* IN TEMPE USING A SPECIFIC PRIMER

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ABSTRACT

Tempe known as a traditional fermented food originated from Indonesia. It has a unique flavour and texture. It also contains high protein and usually serves to substitute meat, fish, or egg as a complement to rice. The manufacture process of Tempe is quite complex and mostly, the traditional process has not employed the hygienic standard. In the process of Tempe making, there are two critical stages of the whole process; i.e. soaking of soybeans and solid state fermentation by ***Rhizopus*** *sp. During the process, foodborne pathogen bacteria such as* ***Escherichia coli*** *could contaminate the product of Tempe. The bacterial contamination could be revealed through culture dependent methods which is costly, laborious, and time consuming. Therefore, the culture-independent method such as polymerase chain reaction using a specific primer could be applied to detect target microorganism to save time and labour. In this study, thirty-one Tempe samples collected from different manufacturers in Semarang, Central Java, Indonesia were analysed by PCR. In order to obtain the bacterial genomic DNA, a modified Chelex 100-Microwave method was employed. The results of DNA extraction showed that the method was an applicable method. It gave high quantity and quality of DNA; therefore, it could be applied in the PCR reaction. The DNA samples were employed in PCR for detection of* ***Escherichia coli*** *using Ecoli706F/R. It was found that 27 out of 31 samples were detected having* ***Escherichia coli*** *contamination showed by the presence of the amplified product size 706 bp. The application of this method could significantly reduce costs and time of analysis in the laboratory. Further response after* ***E. coli*** *were detected could be employed, including investigation of the critical factors in Tempe manufacturing process which allowed E. coli contamination.*

Key Word: Escherichia coli*, rapid detection, PCR, Tempe*

INTRODUCTION

Tempe is a traditional fermented food originated from Indonesia. It has high protein content and serves as a complement to rice, so that it can partially substitute meat, eggs or fish. The manufacture process of Tempe 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 with *Rhizopus oligosporus* (Nout and Kiers, 2005). In Indonesia, there are many Tempe manufacturers spread all over the country area. Based on the data of Indonesian National Standard (SNI), only 5% manufacturers have fulfilled the standard procedure of hygienic Tempe production. The hygienic standard could be revealed from the taste and aroma, as well as the foodborne pathogen bacteria such as *Escherichia coli* and *Salmonella spp.* According to the SNI rules established in 2009, the limit of contamination by *Escherichia coli* is 10 cfu/mL and *Salmonella spp.* is 0 cfu/mL.

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. *Escherichia coli, Listeria monocytogenes,* and *Salmonella spp.* are three kinds of the most important pathogens which could contaminate the fermentation process of Tempe making. Although *Escherichia coli* is the predominant facultative anaerobe of human colonic flora, some strains are responsible for enteric disease (Bischoff *et al.*, 2005; Vergis *et al.*, 2013).

Bacterial contamination could be revealed partially through culture dependent methods. The culture dependent method is costly, laborious, and time consuming. It can hardly be overemphasized the need for the method to detect multiple pathogens simultaneously through a rapid and simple test that can substantially cut down the time, labor, and overall cost of the detection process. PCR based methods are used widely to identify bacteria either as a complemented or an alternative method to phenotypic ways. Basically, DNA sequence is invariant throughout the microbial life cycle and after shortterm environmental stress, therefore, PCR method is more precise. Besides enhancing the sensitivity and specificity of the detection, these methods do reduce much inherent subjectivity in interpreting morphological and biological data. In regard to these, they are considered as superior alternative methods to conventional microbiological/biochemical ways.

Appropriate DNA extraction is an essential tool to obtain reliable data from a culture independent method. Therefore, successful amplicon production first requires an effective DNA extraction and purification method that is easy to use and applicable to a large number of samples. Of the many bacterial genomic DNA (gDNA) extraction and purification methods, some of the most frequently used are the cetyl trimethyl ammonium bromide (CTAB)-phenol-chloroform-isoamyl alcohol method (Cheng and Jiang, 2006; Stefanova *et al.*, 2013), the Chelex 100 method (Martin-Platero *et al.*, 2010; Omar *et al.*, 2014), the colony PCR method (Radha, *et al.* 2013), and the use of microwave ovens (Bastian and Ursula, 2013).

All involve long gDNA extraction times and can co-purify contaminants that affect PCR efficiency such as phenol, chloroform or isoamylic alcohol remnants and some bacterial metabolites (Nechvatal *et al.*, 2008). This makes gDNA purification from large quantities of colonies a time-consuming and inefficient process that is not applicable to all species. In an effort to devise a rapid DNA extraction method, we used a gDNA extraction method by combining and modifying the Chelex 100 and microwave methods, the so-called Chelex 100-Microwave method. This alternative protocol allows extraction of gDNA from food samples in less than 20 min. In this study, we employed a modified Chelex 100-Microwave method for the purification of bacterial genomic DNA in Tempe.

MATERIALS AND METHOD

Sample collection

Thirty-one samples of Tempe were collected from different manufacturers in Krobokan and Tandang Semarang, Central Java, Indonesia. The samples were collected using random sampling techniques. *Escherichia coli* as the positive control and *Salmonella spp.* were obtained from Laboratory of Molecular Biology, Department of Biology, Semarang State University, Indonesia.

Sample treatment

Twenty five gram Tempe were homogenized in 225 ml NaCl 0.9% for 1 min. The mixture obtained were centrifuged for 1 min at 800 x g and the supernatant were moved into new tubes and centrifuged again at 13,000 x g for 5 min. Supernatant were discarded and the pellet was washed with TE pH 8.0 prior to DNA extraction.

Chelex 100-microwave method for genomic DNA purification

The cells were resuspended in 50 µl TES lysis buffer (10 mM TRISBase, pH 7.5; 1 mM EDTA, pH 8.0; 0.5% SDS), and placed in a microwave oven for 10 s at 625 W (Power Level 6). Immediately thereafter, 150 µg proteinase K and 20 µg RNase A were added to the suspension; this was microwaved again under the above conditions. After lysis, the suspension was incubated for 2 min at room temperature, and 150 µl of TE buffer with 25 mg of Chelex 100 were added, and then microwaved again under the above conditions. The sample was then centrifuged at 12,000 x g for 5 min at 4°C. The supernatant containing the gDNA was recovered and quantified. The purified DNA was then used either in PCR reactions (2 µl per reaction) or concentrated by precipitation with 10% of 3 M sodium acetate and 2.5 volumes of 95% ethanol. Finally, the DNA was rinsed twice with 1 ml 70% ethanol, dried at room temperature and resuspended in 200 µl deionized water (Escogido *et al.*, 2010).

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. Spectrophotometer was used for this analysis.

PCR condition

Specific primer Ecoli706-F/R (Yuan *et al.*, 2009) was applied to detect *Escherichia coli.* The primer contains universal sequence CCTTCCTTCCTTCCCCCC on 5'. The primer details are shown in Table 1.

Table 1. Primer Details

Organism	Primer name	Primer Sequence	PCR product (bp)
Escherichia coli	Ecoli706-F	CCTTCCTTCCTTCCCCCCACCTGCGTTGCGTAAATA	706
	Ecoli706-R	CCTTCCTTCCTTCCCCCCGGGCGGGAGAAGTTGATG	

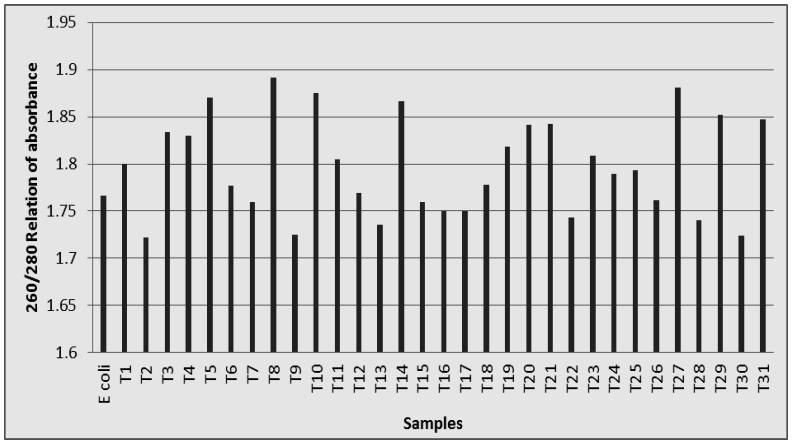

Figure 1. The purity of the gDNA was established from the 260/280 nm ratios.

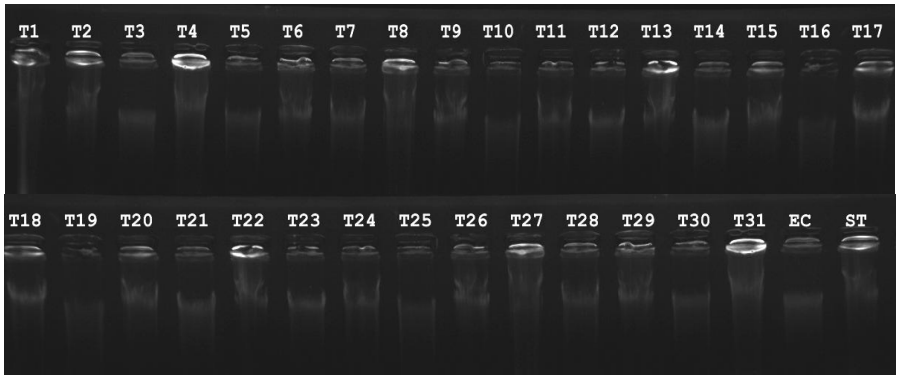

Figure 2. The visualization of the DNA samples isolated from Tempe in the 0.8% agarose gel electrophoresis. T1-T31: Sample number T1-T31; EC: *E. coli*; ST: *Salmonella spp.*

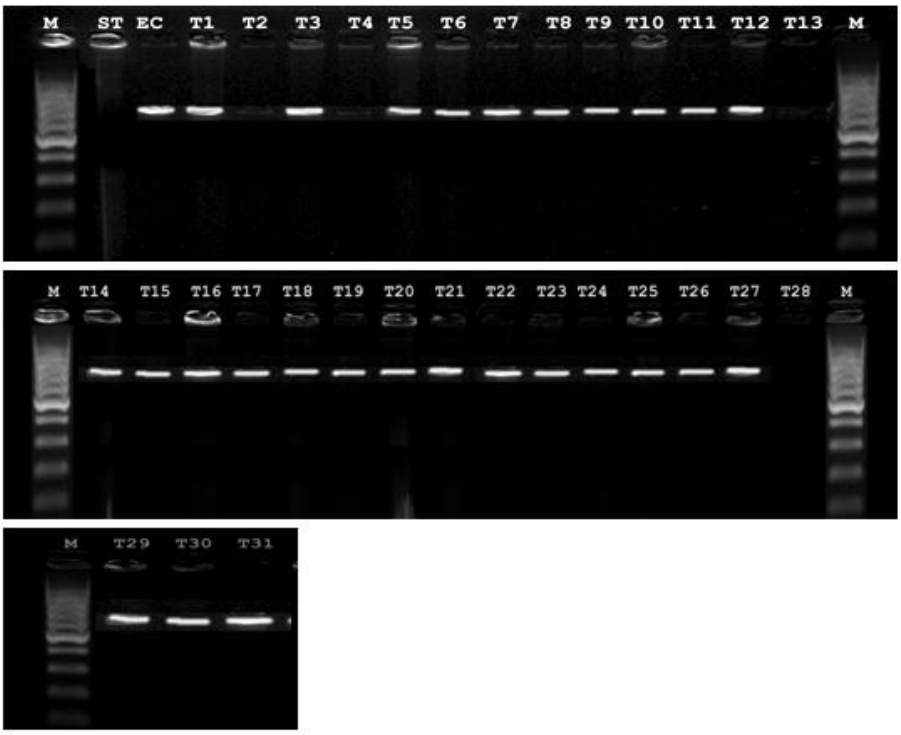

Figure 3. The visualization of the PCR amplification product using Ecoli706-F/R primer in 1.2% agarose gel electrophoresis. M: DNA marker (100bp), T1-T31: Sample number T1-T31; EC: *E. coli*; ST: *Salmonella spp.* Twenty-seven out of thirty-one samples was identified to have *E. coli* contamination as shown by 706 bp PCR product. Four samples and the negative control *Salmonella* sp. did not show any positive band.

In this study, the final optimized concentration of MgCl2 was 1.8 mM, optimized PCR system contained 1.8 mmol/L magnesium chloride, 200 µmol/L dNTPs and 1.5 units of Taq DNA polymerase, 200 nmol/L of each compound specific primer. The final thermal cycling program included an initial 5 min denaturation at 94 ◦C; and then 35 cycles of 30 s at 94 ◦C, 30 s at 60 ◦C, and 50 s at 72 ◦C, followed by a final extension for 10 min at 72 ◦C. Twenty microliters of PCR products were separated by electrophoresis in 20 g/L agarose in 1 × TAE containing 0.2 µg /mL ethidium bromide. Images were recorded with a UV gel documentation system.

RESULTS

The gDNA concentration attained with the Chelex 100-Microwave method from Tempe samples showed a higher purity (average 260/280 ratio = 1.75-2.0). The result of gDNA concentration from 31 samples and *Escherichia coli* are shown in the Figure 1.

The extracted DNA of the samples was measured qualitative using agarose gel electrophoresis. The results are shown in Figure 2.

The results showed that the PCR method using the primers Ecoli706-F/R using the primers could detect *Escherichia coli* indicated by the results of amplification of DNA fragments 706 base pairs. Twenty three out of thirty one samples were detected having contamination of *Escherichia coli* as shown in Figure 3.

The positive samples were generated by DNA fragments of size of about 706 base pairs indicated that the sample was positive containing *Escherichia coli*. The sam-

ples which were not provide DNA bands in the size of 706 base pairs may not contain *Escherichia coli* or were not amplified (Figure 3). The *Escherichia coli* as a positive control apparently produced bands at a size of about 706 base pairs. The negative control using *Salmonella typhi* did not produce the positive band. Based on the previous studies, the primers Ecoli706-F/R specificity was an excellent primer which could be used to detect all strains of both pathogenic and non-pathogenic *Escherichia coli*.

DISCUSSION

Genomic DNA yields and purity

The Chelex 100-Microwave method is a rapid method that extracts high pure genomic DNA without the need or additional purification steps. This method combines the Chelex 100 method for extracting genomic DNA and microwave oven method modifications with addition of proteinase K and RNase A.

The extracted DNA of the samples was measured qualitative using agarose gel electrophoresis. The results are shown in Figure 2. Based on the qualitative and quantitative results of DNA extraction, the Chelex 100-Microwave method has some major advantages, i.e. the gDNA obtained had no detectable contaminants such as phenol or cellular remnants; the gDNA concentration was high; the physicochemical characteristics of the Chelex100 eliminates any peptides and other molecules that might bind to the genomic DNA; and the use of the microwave oven results in extraction of genomic DNA in less than 20 min. This may be because cellular lysis in this method is caused by the rotation and friction of cell molecules when in the microwave oven (Ling *et al.*, 1991; Kim *et al.*, 2009), and not by enzymatic reaction alone. In conclusion, the Chelex 100-Microwave method is a rapid, low-cost and efficient protocol that extracts large quantities of highly pure gDNA that co-purifies no detectable organic solvent remnants.

PCR Analysis

Based on PCR product, in comparison with the examination using the conventional method, the PCR method is more rapid. The agar plating and enrichment to identify *Escherichia coli* in the sample require a longer time compared with the PCR method. In addition, this conventional method has some backwards. First, microorganisms contained in the samples are usually in very small quantities which causing errors in the sampling or in the calculation of the number of microorganisms. Second, sometimes it is difficult to grow the uncultured microorganisms, even using the selective-differential media. Using the PCR method, the identification needs 24-48 hours (Bhunia, 2010).

Analysis of the results of amplification was performed by agarose gel electrophoresis which acts as an electrical circuit to separating DNA fragments based on the number of constituent nucleotides. The smaller size of nucleotide base pairs, the easier them to migrate. The DNA bands formed were observed with UV trans-illuminator tools. The fragment size determination was done by comparing the mobility of DNA fragments with DNA standards of known size. The visualization of DNA in the electrophoresis is more easily done using dyes which could fluorescent, i.e. ethidium bromide (EtBr). EtBr is a planar molecule that can inserts in between DNA bases bond (Sambrook *et al.*, 2001).

The identification of *Escherichia coli* using the conventional method through a series of biochemical tests in the inoculation media and using a confirmatory test by differential and selective media apparently required considerable time tends to be a difficult procedure. This is because the bacteria colonies suspected as *Escherichia coli* in selective as well as differential media often impure and mixed with other Enterobacteriaceae bacteria. In order to get the proper identification, the colony will be identified to rigorously pure so it could take several days to isolate and identify it. Usually, the identification of *Escherichia coli* with conventional methods takes 6 days, whereas the direct PCR method requires only 48 hours. Thus, the PCR method used in this study was faster when compared to the conventional methods.

Tempe Production and Its challenge

Tempe is a traditional fermented food in Indonesia and known for its attractive flavor, texture and superior digestibility. Outside Indonesia, Tempe has become increasingly popular as a nutritious non-meat protein food, for example in the USA, the Netherlands and Japan. There is good suggestion that Tempe is not the product of pure culture fermentation with *R. oligosporus*, and many bacterial species are essential components of the fermentation. *Escherichia coli* are Gram negative pathogenic bacteria which can cause diarrhea. It produces enterotoxin which could affect the absorbance of the water in the digestive tract (Volk and Wheeler, 1990). The existence of *Escherichia coli* in food indicates that the process of manufacturing have not fulfilled the hygienic standard. The hygienic standard component included the employer, the tools, the manufacturing process, the water source and the environment (Bintari and Maskar, 2012). The hygienic status of Tempe is an important requirement to be accepted as a potential food. In Indonesia, the manufacturing process of Tempe is using a traditional way. Some manufacturers have not concerned with the importance of proper sanitation and the hygienic standard. Thus, the contamination of foodborne pathogen and heavy metals are

possible to migrate in the soybean and Tempe itself. 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). 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. Since Tempe is a popular food in Indonesia, it is important to give the understanding of hygienic standard and mind set changing for the manufacturers and the employyer in order to achieve the best quality of Tempe.

Based on this study it can be concluded that method using primer Ecoli706-F/R could be used to detect *Escherichia coli* in Tempe samples. The use of this PCR method sized DNA fragments of 706 base pairs. on The PCR method could detect *Escherichia coli* in Tempe samples faster when compared with conventional methods.

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