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Genetic Diversity of *Klebsiella* spp. Isolated from Tempe based on Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR)

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Tempe is an Indonesian fermented food prepared by fermenting dehulled cooked soybeans with *Rhizopus oligosporus*. Many types of bacteria are also involved during tempe fermentation, and one of these is *Klebsiella* spp. Some isolates of *K. pneumoniae* produces vitamin B12 in tempe but it has also been classified as an opportunistic pathogen. For this reason *Klebsiella* spp. in tempe is important to be studied. The aim of this study was to investigate the genetic diversity of *Klebsiella* spp. from tempe employing ERIC-PCR method. Sixty-one isolates of *Klebsiella* have been isolated from sixteen tempe producers in Bogor, Jakarta, Malang, Tengerang, Bandung and Cianjur. 63F and 1387R primers were used to amplify 16S rDNA sequences, and 1R and 1F primers were used for ERIC analysis. The results of this research showed that sixty-one strains of *Klebsiella* were clustered into 17 groups. Based on ERIC-PCR analysis, isolates of *Klebsiella* could be grouped into different profiles which some of these groups consisted of isolates with identical ERIC-PCR profiles. Several identical ERIC-PCR profiles were found in tempe from the same producer. There was no correlation observed between genetic similarity among isolates with the origin of tempe.

Keywords: Tempe, *Klebsiella*, ERIC-PCR, vitamin B12

INTRODUCTION

Tempe is a traditional fermented soybean food from Indonesia, and it is an important protein source for Indonesian. Tempe is good for health because it contains essential compounds such as isoflavone (Ikehata *et al*. 1968) and vitamin B12 (Keuth & Bisping 1993; Wiesel *et al*. 1997). Liem *et al*. (1977) reported that Indonesian tempe, a protein-rich vegetarian food, is one of the world's first meat analogs. Therefore, tempe has been popular in Japan, USA, Australia, and Europe (Aderibigbe & Osegboun 2006).

Generally, vitamin B12 can only be found in foods derived from animals, therefore vitamin B12 in tempe is essential as a resource of vitamin B12 for certain people such as vegetarians. One type of bacteria that has an ability to synthesize vitamin B12 in tempe is *K. pneumoniae* (Keuth & Bisping 1993). It has been reported that *Klebsiella* sp. in tempe can achieve aproximately 108 CFU/g (Mulyowidarso *et al*. 1989; Mulyowidarso *et al*. 1990; Barus *et al*. 2008).

The presence of *K. pneumoniae* in tempe is advantageous because it produces vitamin B12. However, it has been reported that *K. pneumoniae* was classified as opportunistic pathogens (Podshun & Ullmann 1998). Since research on the characteristics of *Klebsiella* spp. in tempe is limited, an additional study needs to be conducted.

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) (Wilson & Sharp 2006) is often used to analyze the diversity of enteric bacteria, such as *Klebsiella* spp. Characteristic of ERIC sequences are 126 bp, non-coding, and conserve (Versalovic *et al*. 1991). The position and number of ERIC sequences in bacteria vary so it can be used as genetic markers to study the diversity of bacterial isolates. Meanwhile, ERIC-PCR method has been successfully analyzed the diversity of different types of bacteria, such as *Mycobacterium tuberculosis* (Sechi *et al*. 1998) and *Vibrio parahaemolyticus* (Khan *et al*. 2002) because its method is rapid, sensitive, and consistent in analyzing bacterial diversity (Sechi *et al*. 1998; Huiyong *et al*. 2008). Therefore, to assess the genetic diversity of *Klebsiella* spp. from tempe in this study, ERIC-PCR method was used. It is expected that the

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results will be used as basis for further analysis of the *Klebsiella* spp. from tempe.

MATERIALS AND METHODS

Screening of *Klebsiella* Isolates from Tempe. Sixty-one isolates of *Klebsiella* have been isolated from tempe which were obtained direcly from sixteen tempe producers in Bogor, Jakarta, Malang, Tengerang, Bandung, and Cianjur. All isolates were tested by morphology (non-motile, rod-shaped, and Gram negative) and biochemical tests (positive on Voges Proskauer, citrate, urea, and lysine decarboxylase test; and negative on indole and methyl red test). Furthermore, the identification was done based on 16S rRNA gene sequences.

Genome Isolation. *Klebsiella* isolates were cultured in Luria-Broth medium (Oxoid, Hampshire, England) overnight at 37 °C. Genome isolation of each isolate was conducted using Genomic Purification Kit (Fermentas, Lithuania) based on the manufacturer's protocol with a slight modification. The modification was in the speed of centrifugation (13,000 x g), and the addition of 500 mL lysis solution. Obtained DNA genome was stored at -20 °C freezer for further usage.

Amplification of 16S rRNA Gene Sequences. Two specific primers were used to amplify 16S rDNA sequences, namely 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*. 1998). Amplification of 16S rRNA genes sequence of *Klebsiella* isolates was performed in GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA). PCR master mix (25 µL) contained 12.5 mL Go Taq (Promega, Madison, USA), 2 mL primer (25 pmol mL-1), 8.5 mL nuclease free water (Promega, Madison, USA), and 2 mL of DNA template. PCR conditions were as follows: pre-denaturation at 94 °C for 5 min, denaturation at 93 °C for 30 s, annealing at 62 °C for 30 seconds, extension at 72 °C for 30 min, and post-extension at 72 °C for 10 min. Denaturation, annealing, and extension were performed in 35 cycles. PCR products were observed using 1.8% electrophoresis agarose gel (Promega, Madison, USA) then stained with ethidium bromide (Sigma-Aldrich, USA) for 15 min and destained for 5 min before visualized on UV transilluminator using 1 kb ladder (Fermentas, Lithuania) as marker. PCR products were then partially sequenced in Macrogen Inc., Republic of Korea.

ERIC Sequence Amplification. Amplification of ERIC sequence of *Klebsiella* isolates was performed in GeneAmp PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) using the universal primers which comprised Primer 1R 5'-ATGTAACGT CCTG G GGATTCAC-3 ' and primer IF 5' AAGTAAGTGACTGGGGTGAGCG-3' (van der Zee *et al*. 2003; Huiyong *et al*. 2008). PCR master mix (25 µL) contained 12.5 mL GoTaq (Promega, USA), 9.5 mL Nuclease-Free Water (Promega, Madison, USA), 1 uL of each primer (25 pmol mL^{-1}), and 1 mL DNA template. PCR conditions were as follows: pre-denaturation 94 °C for 2.5 min, 94 °C denaturation for 30 s, annealing 47 °C for 1 min, extension 72 °C for 1 min, post-extension 72 °C for 4 minutes, and hold 4 °C. Denaturation, annealing, and extension were performed in 35 cycles. PCR products were observed using 1.8 % electrophoresis agarose gel (Promega, Madison, USA), stained with ethidium bromide (Sigma-Aldrich, USA) for 15 min, and then destained for 5 min. UV transilluminator was used to visualize DNA in gel electrophoresis using 1 kb ladder (Fermentas, Lithuania) as a marker. Based on binary data from profile of ERIC, phylogenetic tree was constructed using MEGA 5 software (Tamura *et al*. 2011).

RESULTS

Sixty-one isolates of *Klebsiella* spp. were randomly selected from tempe which was taken directly from many tempe producers (Table 1). The coloni isolates of *Klebsiella* were pink mucoid on media MCA, non-motile, rod-shaped, and Gram-negative. Based on biochemical tests, the isolates of *Klebsiella* were positive on Voges Proskauer, citrate, urea, and lysine decarboxylase test; and negative on indole and methyl red test.

Table 1. Sixty-one isolates of *Klebsiella* from tempe

Isolate code (Origin of tempe)
1, 2, 3, 4, 5a, 8, 9, 10a, 11a, 12, 13, 14, 15, 255, 339 (Jakarta)
5b, 7b, 10b, 11b, 17b, 165, 175, 194, 198 (Malang)
108, 110, 115, 120, 133, 135, 140, 145, 157, 210, 211, 215, 219, 221, 225, 226, 233, 240, 245 (Bogor)
16, 17a, 18, 19, 52, 59, 305 (Bandung)
20, 21, 22, 23, 24, 25, 248, 250, 49, 50, 51 (Cianjur)