MICROBIOLOGY INDONESIA
ISSN 1978-3477

Volume 4, Number 1, April 2010
p 1-5

Genetic Diversity of Yeasts from Fermented Orange Juice Based on PCR-RFLP and Sequence Analysis of the Internal Transcribed Spacer Regions

SUSAN SOKA* AND ANASTASIA SUSANTO

Faculty of Biotechnology, Universitas Katolik Indonesia Atma Jaya,
Jalan Jenderal Sudirman 51, Jakarta 12930

Orange is one of the most valuable and common fruits in Indonesia. High glucose level in orange juice provides good growth conditions for yeasts. In this study, yeasts were isolated from fermented orange juice and subjected to diversity analysis. The analysis was conducted using restriction fragment length polymorphism on the internal transcribed spacer (ITS) region (including ITS1, 5.8S rRNA gene and ITS2), which was amplified using PCR with ITS1 and ITS4 primers. Restriction enzymes used in this research were *Hha*I, *Hinf*I and *Hae*III. A total of 24 yeast isolates were obtained from three different kinds of fermented orange juices (Indonesian Medan orange, Sunkist orange and Indonesian Pontianak orange). RFLP analysis of ITS regions revealed different amplified PCR fragment sizes and restriction profiles for each type of orange juice. However, all yeasts isolated from the same type of orange juice showed identical restriction patterns. Sequencing of ITS regions showed that three different yeast species were detected from each type of orange, e.g. *Pichia veronae* from Indonesian Pontianak orange, *Cryptococcus albidosimilis* from Sunkist orange and *Issatchenkia orientalis* from Indonesian Medan orange.

Key words: orange yeasts, 5.8S-ITS region, RFLP

Orange juice contains high levels of sugar and a low pH range (3-4). Such conditions are optimal for lactic acid bacteria, mold and yeast growth. Lactic acid bacteria are the main contaminants in fruit juices, but the growth of these bacteria may be inhibited by pasteurization, high sugar concentration and low temperature storage (Arias *et al.* 2002).

Yeast is a unicellular fungus that is tolerant to high osmotic pressure and low pH (Okada *et al.* 2006), as well as able to grow at low temperature (4-10 ^{o}C), and has a wide range of habitats. The presence of certain yeast species will result in orange juice spoilage (Thomas and Davenport 1985).

Previously, yeasts were identified based on their morphological and physiological characteristics. This conventional method is laborious and time-consuming (Arias *et al.* 2002). Since the 1990's, many PCR-based methods have been used to identify yeasts, including Restriction-Fragment-Length-Polymorphism (RFLP) analysis of Internal Transcribed Spacer (ITS) regions (Arias *et al.* 2002). RFLP analysis begins with amplification of the internal transcribed spacer (ITS) regions (including ITS1, 5.8S rRNA gene and ITS2), using PCR, followed by restriction endonuclease digestion.

ITS regions in fungal nuclear DNA have been used for yeast identification. This method is the most effective as a site in analyzing interspecies, as well as intraspecies, of yeasts. One unit of ribosomal DNA comprises of 5.8S rDNA, ITS1 and ITS2 (non-coding region), small subunit DNA (SSU) and large subunit DNA (LSU) (Heras-Vasquez *et al.* 2003). ITS1 lies in between 18S rDNA (in SSU) and 5.8S rDNA, while ITS2 lies in between 5.8S rDNA and 28S rDNA (in LSU) (James *et al.* 1996). ITS regions have higher varieties compared to other rDNA regions, such as SSU and LSU (Vilgalys *et al.* 1994). Therefore, ITS regions are the main site for identification analysis of yeasts, generally using ITS1 and ITS4 primers.

In this research, a number of yeast isolates were obtained from fermented orange juice. These isolates were then identified based on PCR-RFLP and the sequence analysis of their ITS regions.

MATERIALS AND METHODS

Yeast Isolation. Yeasts were isolated from three types of orange, Indonesian Pontianak orange, Sunkist orange and Medan orange. A group of six oranges was used for each orange type. Each orange was washed and pasteurized in a waterbath at 80^{o}C for two minutes. Afterwards, the juice of the orange was squeezed out and transferred into a flask and further fermented in a shaking waterbath at 37^{o}C and 120 rpm for three days. The resulting fermented orange juices were undiluted and diluted to 10^{-2}, 10^{-4} and 10^{-6} in sterile distilled water. As much as 100 µL of each dilution was spread onto yeast peptone D-glucose (YPD) agar (1% w/v yeast extract, 2% w/v bactopeptone, 2% w/v D-glucose and 2% w/v bactoagar) (Heras-Vazquez *et al.* 2003) supplemented with 50 µg mL^{-1} chloramphenicol to inhibit bacterial growth and incubated at 30^{o}C for three days. As many as eight single yeast isolates from each type of orange were streaked onto YPD agar containing chloramphenicol.

Yeast DNA Extraction. Yeast isolates were grown in YPD medium at 30^{o}C for three days. A loop of each colony was suspended in a mix of 200 µL TE buffer pH 8.0, 200 µL P:C:I (25:24:1), and 0.65 g glass beads (Sigma G1152-100G)). The mixture was vortexed for three minutes, followed by centrifugation at 16 000 x g for 10 minutes. Afterwards, 20 µL supernatant was transferred into a fresh

*Corresponding author: Phone.: +62-21-5731740,
Fax: +62-21-5719060, E-mail: susan.soka@atmajaya.ac.id

microtube. Pelleted nuclear DNA was suspended in TE 1X buffer pH 8.0 and stored in -20°C prior to use.

Amplification of 5.8S rRNA-ITS Site. The 5.8S rRNA-ITS site was amplified using Gene Amp® PCR System 2400 (Perkin Elmer, USA) and two primers, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC YCC GCT TAT TGA TAT GC-3') as described by Heras-Vasquez *et al.* (2003). The PCR reaction consisted of: 500 ng sample DNA, 1.0 µL 10 mM dNTP mix, 25 µM ITS1 primer, 25 µM ITS4 primer, 25 units Taq polymerase (New England Biolabs), 4 µL Taq polymerase buffer and combined with sterile double-distiled H_2O give up to 50 µL of reaction mixture. PCR reactions were run at 95°C for 5 min; 35 cycles of 94°C for 1 min, 44°C for 2 min, and 72°C for 2 min; and 72°C for 10 min. PCR products were visualized on 1.5% w/v agarose gel using ethidium bromide staining.

Restriction Fragment Length Polymorphism (RFLP) Analysis of 5.8S rRNA-ITS Site. As much as 10 µL of each amplicon was digested using one unit of *Hae*III, *Hha*I and *Hinf*I restriction endonuclease in a total of 20 µL reaction volume at 37°C overnight. Restriction patterns were visualized on 3% (w/v) agarose gel using ethidium bromide staining.

DNA Sequencing. Amplicons were purified using HiYield™ Gel/PCR DNA Extraction Kit (Real Biotech Center, Taiwan) and subjected to cycle sequencing using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA) following the manufacturer's instructions. Cycle sequencing product purification was performed using an ethanol-EDTA precipitation method (Sambrook and Russel 2001). Sequencing was conducted on an ABI Prism 310 Genetic Analyzer (Applied Biosystem, USA) system. The obtained DNA sequences were compared to GenBank database using BLASTN (www.ebi.ac.uk) and were deposited in Genbank.

RESULTS

A total of 24 yeast isolates were obtained from three types of orange. Isolates from Indonesian Pontianak orange and Sunkist orange showed similar phenotypic characteristics; the yeast colonies were round, milky white and mucoid; while the margin of yeast colonies from Indonesian Medan orange are entire and white in color.

The 5.8S-ITS site of all of the yeast isolates was amplified using ITS1 and ITS4 primer. Indonesian Pontianak orange revealed a 600 bp amplified product, while Sunkist orange and Indonesian Medan orange each revealed a 650 bp and a 500 bp product respectively (Fig 1).

Amplified 5.8S-ITS-site-digestion using *Hha*I revealed two 300 bp fragments for Indonesian Pontianak orange samples, two 300 bp fragments for Sunkist orange samples, and two 200 bp fragments and a 50 bp fragment on Indonesian Medan orange samples (Fig 2). Meanwhile, amplified 5.8S-ITS-site-digestion using *Hinf*I showed two 300 bp fragments for Indonesian Pontianak orange samples, 350 bp and 280 bp fragments for Sunkist orange samples, as well as 250 bp and 180 bp fragments for Indonesian

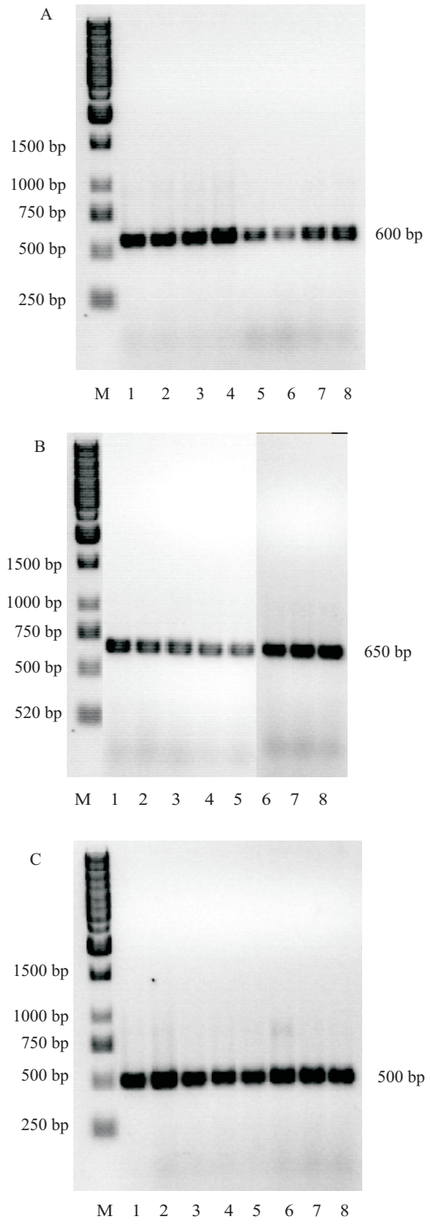

Fig. 1 Amplification of yeast ITS regions from orange juice. A, Indonesian Pontianak orange, 600 bp; B, Sunkist orange, 650 bp; C, Medan orange, 500 bp. M: 1kb lambda-ladder DNA marker (Fermentas); 1-8: isolate 1-8.

Medan orange (Fig 3). *Hae*III digestion on the same amplified products revealed a 480 bp and 70bp fragments for Indonesian Pontianak orange samples, a 500 bp, 70 bp and 60 bp fragments for Sunkist orange samples, and a 400 bp, 80 bp and 20 bp fragments for Indonesian Medan orange (Fig 4). The size of 5.8S-ITS amplification products and

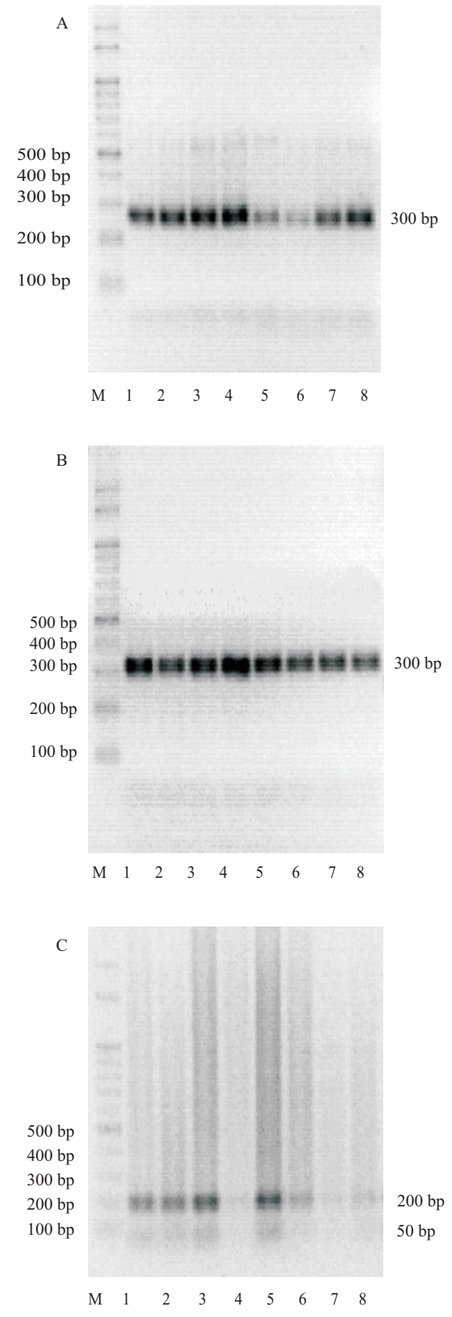

Fig. 2 DNA digestion profile using *Hha*I from each orange samples. A, Indonesian Pontianak orange; B, Sunkist orange; C, Indonesian Medan orange. M: 100bp ladder DNA (RBC-Taiwan); 1-8: isolate 1-8.

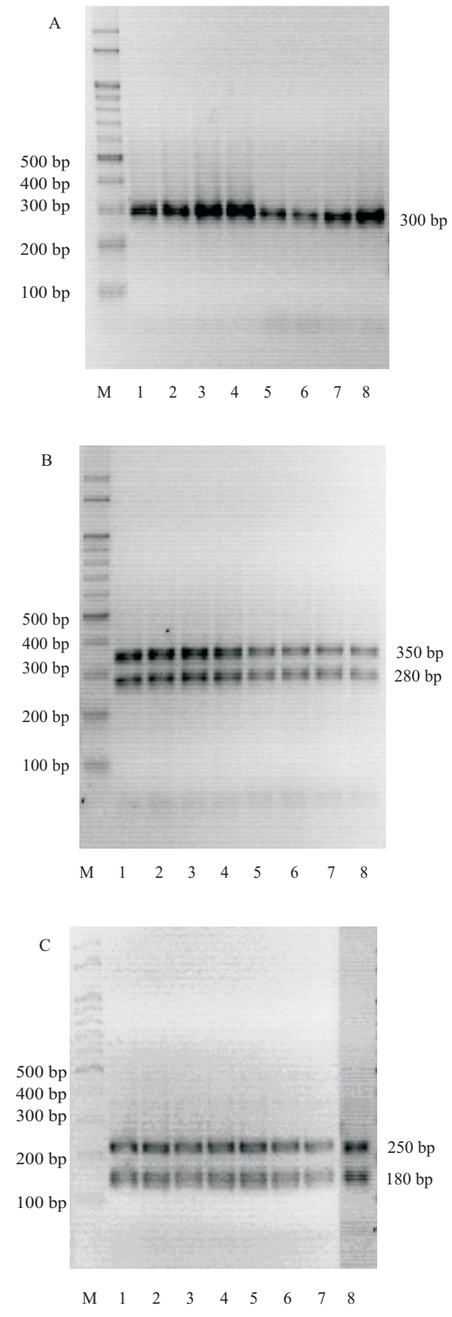

Fig. 3 DNA digestion profile using *Hinf*I from each orange samples. A, Indonesian Pontianak orange; B, Sunkist orange; C, Indonesian Medan orange. M: 100bp ladder DNA marker (RBC, Taiwan); 1-8: isolate 1-8.

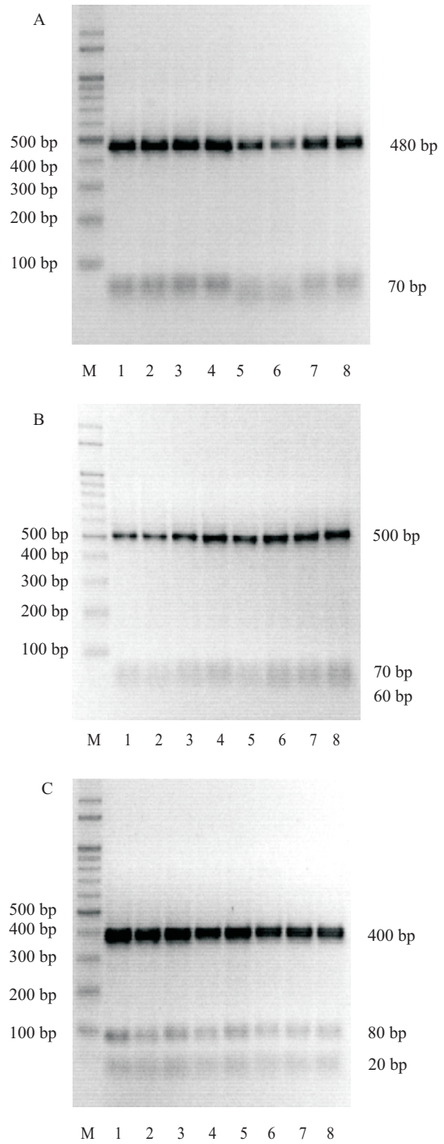

Fig. 4 DNA digestion profile using *Hae*III from each orange samples. A, Indonesian Pontianak orange; B, Sunkist orange; C, Indonesian Medan orange. M: 100 bp ladder DNA marker (RBC, Taiwan); 1-8: isolate 1-8.

their restriction fragments is summarized in Table 1. In the majority cases, the restriction fragments present identical molecular sizes. It may be assumed that single band present fragments with similar molecular sizes.

DISCUSSION

Orange juices are acidic beverages with high sugar content. Under this condition, acidolactic bacteria, molds and yeast comprise the typical microbiota present in orange juices. Yeasts tolerate high-osmotic and low-pH conditions and grow at broad temperatures and therefore cause spoilage in the processed product (Arias *et al.* 2002).

The most widespread methods for the detection of wild yeasts still rely upon the use of various selective solid media (Jespersen and Jakobsen 1996). The main disadvantages of this technique are the long period before obtaining results and the inaccuracy of results. Molecular biology techniques provide several alternative methods, which are timesaving and more accurate (Barszczewski and Robak 2004). Hence, they have become increasingly important tools in solving industrial problems (Loureiro and Querol 1999). Restriction-Fragment-Length-Polymorphism (RFLP) analysis of Internal Transcribed Spacer (ITS) regions was successfully used for the identification of yeasts species (Esteve-Zarzoso *et al.* 1999).

While yeasts isolated from different types of orange showed various digestion profiles, the isolates obtained in each orange revealed similar digestion profiles (Fig 2-4). In contrast to fresh orange juice, after fermentation there is a possibility of domination by a particular species of yeast due to the inhibition of the growth of certain species during fermentation (Arias *et al.* 2002).

Amplifed ITS regions sequence analysis was also performed to identify the species of yeast isolates. BLASTN analysis showed different similiarities for each type of orange; *Pichia veronae* from Indonesian Pontianak orange, *Cryptococcus albidosimilis* from Sunkist orange, and *Issatchenkia orientalis* from Indonesian Medan orange. *P. veronae* has a beady texture, is teleomorphic and is generally found in plants. *C. albidosimilis* was identified for the first time in the Antartic and it reproduces through bipolar budding (Scorzetti *et al.* 2004). *Issatchenkia orientalis*, a teleomorph of *Candida krusei*, is a common habitant of rotting oranges (Tuset and Perucho 2000). The DNA sequences of the yeast have been deposited in Genbank. The Genbank accession numbers are Eu812228 for *P. veronae*, EU812229 for *C. albidosimilis*, and EU812230 for *I. orientalis*.

Table 1 PCR amplification products and restruction fragments of yeasts isolated from three types of orange using *Hha* I, HinfI and *HaeIII*

Types of Orange	PCR Fragments (bp)	Restriction Fragments (bp)		
		Hha I	*Hinf*I	*Hae*III
Indonesian Pontianak Orange	600	300 + 300	300 + 300	480 + 70
Sunkist Orange	650	300 + 300	350 + 280	500 + 70 + 60
Indonesian Medan Orange	500	200 + 200 + 50	250 + 180	400 + 80 + 20

As many as 24 yeast isolates were obtained from Indonesian Pontianak, Sunkist, and Indonesian Medan orange. Three different yeast species were identified from these types of oranges, including *P. veronae* from Pontianak orange, *C. albidosimilis* from Sunkist orange, and *I. orientalis* from Medan orange. The genetic diversity of these species was analyzed using RFLP and further identified by sequencing. From both analyses, we concluded that the fermentation process of each orange juice was dominated by one particular species of yeast.

ACKNOWLEDGMENT

This study was supported by Atma Jaya Catholic University Research Institute.

REFERENCES

Arias CR, Burns JK, Friedrich LM, Goodrich RM, Parish ME. 2002. Yeast species associated with orange juice: Evaluation of different identification methods. Appl Environ Microbiol 68:1955-61.

Barszczewski W, Robak M. 2004. Differentiation of contaminating yeasts in brewery by PCR-based techniques. Food Microbiol 21:227-231.

Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int J Syst Bacteriol 49:329-337.

Heras-Vasquez FJL, Mingorance-Cazorla L, Clemente-Jimenez JM, Rodriguez-Vico F. 2003. Identification of yeasts species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers. FEMS Yeast Res 3:3-9.

James SA, Collins MD, Roberts IN. 1996. Use of an rRNA Internal Transcribed Spacer Region to Distinguish Phylogenetically Closely Related Species of the Genera *Zygosaccharomyces* and *Torulaspora*. Int J Sys Bacteriol 46:189-94.

Jespersen L, Jakobsen M. 1996. Specific spoilage organisms in breweries and laboratory media for their detection. Int J Food Microbiol 33: 139-55.

Loureiro V, Querol A. 1999. The prevalence and control of spoilage yeasts in food and beverages. Trends Food Sci Technol 10:356-65.

Okada K, Ravi H, Smith EM, Goode BL. 2006. Aip1 and cofilin promote rapid turnover of yeast actin patches and cables: a coordinated mechanism for severing and capping filaments. Mol Biol Cell 17:2855-68.

Sambrook J, Russel D. 2001. Molecular Cloning: A Laboratory Manual. 3rd Ed. New York: Cold Spring Harbor Laboratory Pr.

Scorzetti G, Petrescu I, Yarrow D, Fell JW. 2004. *Cryptococcus adeliensis* sp. nov., a xylanase producing basidiomycetous yeast from Antarctica. Antonie van Leewenhoek 77:153-7.

Thomas S, Davenport RR. 1985. *Zygosaccharomyces bailii*: a profile of characteristics and spoilage activities. Food Microbiol 2:157-69.

Tuset JJ, Perucho R. 2000. First Recognition of *Issatchenkia orientalis* in mature citrus fruits in Spain. Plant Dis 85:335.

Vilgalys R, Hopple JS, Hibbett DS. 1994. Phylogenetic implications of generic concepts in fungal taxonomy: The impact of molecular systematic studies. Mycol Helvet 6:73-91.