

*Full Length Research Paper*

# Genetic diversity of yeasts from *Ragi tape* “starter for cassava and glutinous rice fermentation from Indonesia” Internal Transcribed Spacer (ITS) region

Tati Barus\* and Steffysia

Abstract

Faculty of Biotechnology, Atma Jaya  
Catholic University of Indonesia, Jalan  
Jenderal Sudirman No.51,  
Jakarta 12930

\*Corresponding Author's E-  
mail: [tati.barus@atmajaya.ac.id](mailto:tati.barus@atmajaya.ac.id)

Tape is a traditional food from Indonesia made from cassava tubers (*Manihot utilisima*) or glutinous rice (*Oriza sativa glutinosa*) as the alternative substrate. It can only be produced through a fermentation process mostly done by yeast and bacteria; therefore, yeast determine the quality of tape largely. In this study, yeasts were isolated from *Ragi tape* and subjected to diversity using restriction fragment length polymorphism (RFLP) on the 5.8S rRNA gene and the two ITS regions. A total of 35 yeast isolates were isolated from eight different types of *Ragi tape*. The results of this research showed that not all *Ragi tape* sold in the market contain yeast. Of the 12 different types of *Ragi tape*, only eight of them contained yeast. Other 35 yeast isolates were selected from eight types of *Ragi tape* for PCR-RFLP analysis. According to the digestion profiles of RFLP, 35 yeast isolates were clustered into 4 groups, as follow: group 1 consisted of 27 isolate (77%), group 2 six isolates (17%), group 3 and group 4 one isolate (3%), respectively. Isolates of group 1 and group 2 were similar to *Pichia jadinii* and *Pichia kudriavzevii*,

**Keywords:** *Ragi tape*, Yeast, PCR-RFLP

## INTRODUCTION

Tape is a fermented food from Indonesia that can be found in all regions of Indonesia. It is a popular fermented food beside tempe. It is produced through a fermentation process mainly by yeast. For traditional fermentation of tape, fresh cassava roots or glutinous rice are boiled 1-2 h until soft, cooled, and mixed with a starter, commonly called *Ragi tape*. After that, it is packaged in a sealed container and fermented. Fermentation takes place within 2-3 days at temperatures of 30-37 °C. Tape can be consumed directly after fermentation is complete or processed further to mix with drinks or other foods.

Tape has a sweet with slightly sour taste, soft texture, watery, and smells like alcohol. Sometimes, the flavor of the tape is not consistent though produced by the same

tape maker and uses the same variety of cassava tuber or glutinous rice. In tape production, the tape maker will mix *Ragi tape* with substrate. *Ragi tape* likely constitutes the main source of microorganism which is active in the later fermentation. Therefore, the quality of tape is determined by quality of microorganism at *Ragi tape*. *Ragi tape* can be found in traditional markets in Indonesia. It is traditionally processed by the same method of from generation to generation. All *Ragi tape* contains raw starch as carrier of microorganisms. Generally, *Ragi tape* is made in home industries, which is then sold with or without trade mark.

The influence of microorganisms on the quality of fermented food production has been widely reported

(Rodas et al., 2003; Broadbent et al., 2002; Stahnke, 1994). Previously, it has been reported that microorganisms in *Ragi tape* are *Weissella spp*, *Enterococcus spp*, *Pediococcus pentosaceus*, *Bacillus sp*, *Clostridium*, *Lactobacillus sp.*, uncultured bacterium, and *Eubacterium* (Sujaya et al., 2010). However, research on yeast and their diversity is still not performed, despite the fact that yeast is a major microorganism in fermentation of tape (Azmi et al., 2010). Without yeast, tape cannot be produced. Information about the diversity of these yeasts can be the basic knowledge to select the best yeast isolate to be a starter.

It has been reported that yeast plays a role in the fermentation of cassava, such as *akyeke* from Africa (Obilie et al., 2003), *tapioka* fermentation from Indonesia (Razip et al., 2006), *lafun* from Africa (Padonou et al., 2009), and *attièkè* from Dabou (Assanvo et al., 2006). The conventional method for identification of yeast using morphological and physiological criteria is not always sufficiently discriminatory for this purpose. Therefore PCR-based methods have been used to identify yeast. One of them is Restriction Fragment Length Polymorphism (RFLP) of 5.8S rRNA gene and the two ribosomal ITS Regions. ITS region displays significant heterogeneity in both length and nucleotide sequences. Both types of variation have been extensively used to distinguish yeast strain and closely related species. It has been reported that the RFLP method as a rapid and easy method is successfully applied to identify yeast (Assanvo et al., 1999). Therefore the objective of this study is to reveal diversity of yeast from *Ragi tape* based on PCR-RFLP of 5.8S rRNA gene and the two ribosomal ITS Regions.

## MATERIALS AND METHODS

### Collection of samples

Twelve samples of *Ragi tape* purchased from traditional markets from different parts of Indonesia i.e from Jakarta 2 samples, Cianjur 2 samples, Yogyakarta 1 sample, Medan 4 samples, Banjarmasin 2 samples, and Banjarnegara 1 sample. All the samples are produced on home industries by different producers. After purchased, the samples were collected in sterile polyethylene sampling bag, transported to the laboratory for further analysis.

### Isolation of yeasts

Representative 10 g portion of *Ragi tape* were homogenized with 90 mL sterile 0.85% w/v NaCl solution and homogenized with vortex with maximum speed (Maxi Mix II, Barnstead). Appropriate decimal dilutions (0.1 mL)

of the homogenates in the same diluent were spread over plates of the Yeast mold agar (YMA) (3 g/L Yeast extract (Oxoid, LP 0021), 3 g/L Malt extract (Oxoid, LP 0039), 5 g/L Bacteriological Peptone (Oxoid, LP 0034); 10 g/L Glucose (Merck, 346351), 20g/L Agar Bacteriological (Oxoid, LP0011)) and incubated at 30 °C for 48 h. Each representatives colony were randomly selected and purified by dilution streaks on the same media. After microscopic examination of morphology the purified cultures were ground on the same media and stored at 4°C prior to used.

### Isolation of yeast genome

The method was based on the protocol of Soka and Susanto (2010) with modifications. Of 35 yeast isolates were cultured on plate of YMA. Incubation was performed at 30°C for 48 h. The colony was suspended by vortexing at high speed in 400 µL 1x TE buffer (10mM Tris-Base (Merck, 648310), 1 mM EDTA (Merck ,137004)) pH 8 and added with 5 units of solid Pyrex glass beads with a size of 4 mm (Sigma, Z143936). The solutions were given freeze-thaw treatment at -4°C and vortexed at high speed for three minutes to lyses the yeasts cell wall. Solution was mixed with P:C:I (25:24:1), vortexed for three minutes, and followed by centrifugation at 16000 x g for 10 minutes. About 300µL supernatant was transferred to a new microtube and added with 300 µL cold absolute isopropanol. The mixture was incubated at -20°C for 20 minutes followed by centrifugation at 16 000 x g for 10 minutes. The pellet was resuspended with 20 µL of 1x TE buffer pH 8. DNA extracts stored in -20°C prior to use.

### Amplification of 5.8s rRNA gene and the two ribosomal ITS regions

The 5.8S rRNA gene and the two ribosomal ITS regions were amplified using 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 Ferreira et al. (2009). All the PCRs were performed 50 µL of reaction mixture containing 25 µL of GoTaq (Promega, A6001), 2 µL of forward and reverse primers, 2 µL of DNA template, and 19 µL of autoclaved deionised water. The amplification was performed with a total of 35 PCR cycles in thermal cycler Gene Amp® PC System 2400 (Perkin Elmer, USA). The cycling program was started with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation of 94 °C for 1 min, annealing at 56 °C for 2 min and elongation at 72 °C for 2 min. The PCR was ended with a final extension at 72 °C for 10 min and the amplified product cooled at 4 °C. The amplified DNA fragment were visualized to 1% (w/v) agarose (Lonza, 50004) gel after staining using ethidium bromide (Sigma,

E7537-5G). DNA marker were included as a standard for the calculation of the size of the DNA fragments.

### PCR-RFLP of 5.8S rRNA gene and the two ribosomal ITS Region

Each DNA product was single digested for 16 h at 37 °C by mixing 10 µL of DNA product with 2 µL of 10X buffer (Fermentas, BR5), 2 µL each of *Hinf*I (Fermentas, ER0801), *Hae*III (Fermentas, ER0151), and *Hha*I (Fermentas, ER01851), then 6 µL sterile deionised water. RFLP products were analyzed by horizontal electrophoresis at 3% (w/v) agarose (Fermentas, R0491) gel. DNA molecular mass marker (1-kb DNA ladder) was used as a DNA molecular size marker. All electrophoreses were carried out at 60 V for 90 min. After electrophoreses, the gels were stained with ethidium bromide (Sigma, E7537-5G) and observed under UV light. To determine series of bands that appeared in the gel as a result of successful restriction, estimation of the DNA fragment size was done using a mathematical equation that links migration rate to molecular weight. The relevant formula  $D = a - b (\log M)$  (Brown 1986) was applied for analysis. D is the distance moved, M is the molecular weight, a and b are constants that depend on electrophoresis condition.

## RESULTS AND DISCUSSION

### Distribution of yeasts in *Ragi* tape

Twelve types of *Ragi* tape purchased from traditional markets in Jakarta, Yogyakarta, Medan, Cianjur, Banjarmasin and Banjarnegara are produced by different producers. In general, *Ragi* tape has a particular color, ranging from white, opaque, and yellow. The shape resembles a flattened round and round tablet that has a diameter of about 2 to 5 cm. Each of these *Ragi* tape has no expired date information. Of the 12 types of *Ragi* tape compiled, only two types of *Ragi* tape packed with closed plastic bags. Otherwise, the rest are unwrapped, sold grain, and will be wrapped with scrap paper or plastic bags only when purchased by consumers.

From this research, it is known that not all *Ragi* tape in the market contains yeast. Of the 12 types of *Ragi* tape collected from different regions, only 8 types contain yeast.

Nevertheless, information about the presence of yeast in *Ragi* tape is, absolutely important because it determines the success of tape production. Tape is generally produced individually on a small scale (home industry) for sale or daily consumption. Tape production cannot always be made successfully especially for people who do not usually produce it because not all *Ragi* tape contains yeast based on the findings of this

research.

There are several possible factors that cause *Ragi* tape sold in traditional markets does not contain yeast. One of them is that the possibility of *Ragi* tape is expired, so the yeast in *Ragi* tape is already dead. This can happen because each type of *Ragi* tape that is produced today does not include expired date. Expired date is important to be informed because the ability of yeast cells to survive in *Ragi* tape has certain period. Yeast which is not found in some types of *Ragi* tape can also occur due to processing factor; that is, *Ragi* tape that is traditionally processed, and there is no controls to ensure whether or not the *Ragi* tape produced contains yeast.

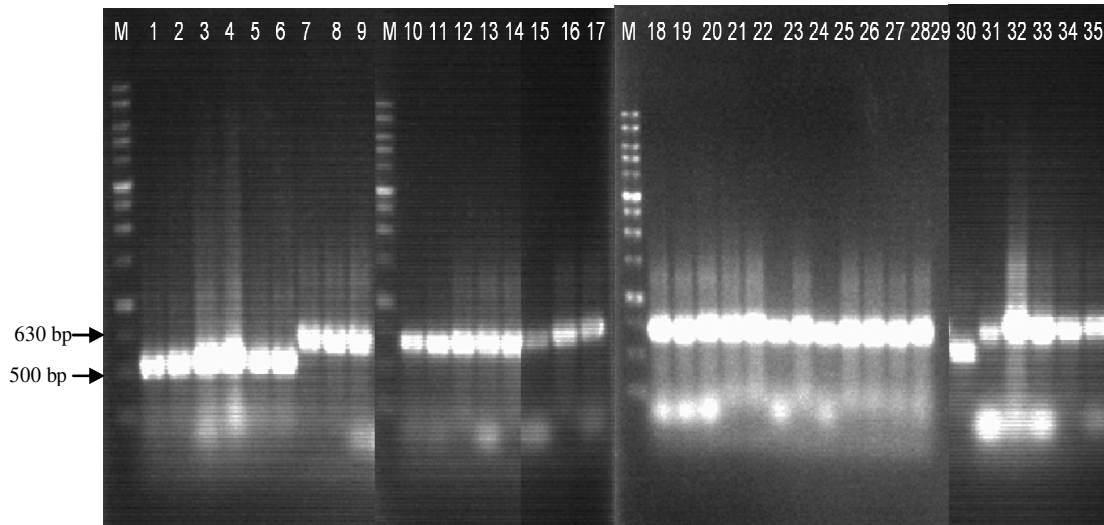
### RFLP of 5.8S rRNA gene and the two ribosomal ITS Region

A total of 35 of 112 yeast isolates were taken to be identified and analyzed their genetic diversity. The 5.8S rRNA gene and the two ribosomal ITS Regions of 35 yeasts isolates were successfully amplified with ITS1-ITS4 primers. In this investigation, primers ITS1-ITS4 were selected because from previous studies reported that they are successfully amplified 5.8S rRNA gene and the two ribosomal ITS Regions of yeasts, yielded distinctly different patterns (Granchi et al., 1999).

After electrophoreses in agarose gel 3%, the amplification products of 35 yeast isolates show two differences in size depending on the yeast isolate. Amount 28 yeast isolates showed the amplicon size in 630 bp, and 7 yeast isolates showed amplicon size in 500 bp (Figure 1). Amplicon size differences may indicate differences in the genus or even species. However, identification based on the amplicon size is not enough. It can be seen in all yeast isolates from Jakarta *Ragi* tape and isolate from Yogyakarta *Ragi* tape (NkIA7 isolate) digestion patterns, which have same amplicon size (500 bp) but show very different digestion patterns using *Hinf*I, *Hae*III, and *Hha*I restriction enzymes. Similarly, NkIB3 with amplicon size 630 bp showed a different digestion pattern using *Hae*III restriction enzyme compared to the others. Thus, this study suggests that differences in amplicon size were not sufficient to distinguish yeast into strain level. However, RFLP analysis can differentiate up to strain level.

Digestion patterns of NkIB3 (Table 1) showed that using two restriction enzymes (*Hinf*I and *Hha*I) have the same digestion patterns compared to the other while using *Hae*III, NkIB3 is different from the other. The quality of the RFLP results can be improved using a larger number of restriction enzymes, but usually no more than five (Ferreira et al. 2009).

The amplicons of all the examined yeast isolates were digested by three differences restriction enzyme *Hinf*I, *Hha*I, and *Hae*III. Profiles of 5.8S rRNA gene and the two ribosomal ITS Regions by the enzymes restriction



**Figure 1.** Amplification of yeast rDNA-ITS Regions from: Jakarta (1 to 6), Medan (7 to 26), Cianjur (27-29), and Yogyakarta (30 to 35). M: a molecular weight marker 1 Kb ladder (Fermentas).

**Table 1.** PCR-RFLP profile from 35 yeast isolates using *HinfI*, *HaeIII*, and *HhaI* restriction enzymes

Region	Total Isolate	Isolate Code	Amplicon Size (bp)	Restriction fragment (bp)		
				<i>HinfI</i>	<i>HaeIII</i>	<i>HhaI</i>
Jakarta	6	CapA1, CapA3, CapA10, CapA15, CapB6, CapB8	500	206+147 +147	360+100	200+200 +50+50
Medan	5	Brs1A1, Brs1A2, Brs1A5, Brs1A6, Brs1A7	630	315+315	530+100	300+300
	4	Brs2B3, Brs2B9, Brs2B10, Brs2B11	630	315+315	530+100	300+300
	3	Brs3B4, Brs3B5, Brs3B6	630	315+315	530+100	300+300
Cianjur	6	Mdn1A6, Mdn1A14, Mdn1A8, Mdn1A9, Mdn1B9, Mdn1B14	630	315+315	530+100	300+300
	2	Mdn2A2, Mdn2B7	630	315+315	530+100	300+300
	3	CiA1, CiA7, CiA13	630	315+315	530+100	300+300
Yogyakarta	6	NkIA7	500	270+230	500	280+ 100+100
		NkIB3	630	315+315	530	300+300
		NkIB12, NkIB16, NkIB18, NkIB22	630	315+315	530+100	300+300

produced 1 to 4 bands with different sizes (Table 1). It was found that of thirty five yeast isolates, 27 yeast isolates (77.14%) have the same digestion patterns shown by two 315 bp fragments using *HinfI*, a 530 bp and 100 bp fragments using *HaeIII*, and two 300 bp fragments using *HhaI*. Six yeasts isolates (17.14%) have the same digestion patterns, a 206 bp and two 147 bp fragments using *HinfI*, a 360 bp and 100 bp fragments using *HaeIII*, and two 200 bp fragments and two 50 bp fragments using

*HhaI*. With the two other yeast isolates (5.72%), each of them showed different digestion patterns compared to other yeast isolates (Table 1). It is important to inform that fragments smaller than 50 bp could not be reproducibly visualized and were not included in this analysis.

Based on RFLP profile with from *HinfI*, *HhaI*, and *HaeIII* restriction enzymes of 5.8S rRNA gene and the two ITS Regions, the 35 isolates of yeast clustered into 4

groups. Group 1 consisted of 27 isolate (77%), group 2 six isolates (17%), and group 3 and group 4 one isolate (3%), respectively. According to the sequences of 5.8S rRNA gene and the two ITS regions that isolates of group 1 and group 2, they were similar to *Pichia jadinii* and *Pichia kudriavzevii*, respectively.

The digestion patterns generated after digestion by restriction enzymes are important in order to reunite isolates in groups according to the size of each fragment, when there is a large number of isolates to be identified. It is possible to reduce total number of samples to be sequenced. Therefore, it is necessary to use a representative number of colonies for each group. It is possible to reduce the costs of identification, since PCR-RFLP is a cheaper technique than DNA sequencing analysis (Weidner et al., 1996).

The results of this study indicate a tendency that every *Ragi tape* that comes from one producer in one region has a similar genetic diversity, as shown in yeast from *Ragi tape* from Jakarta, Medan, and Cianjur (Table 1). In each of the *Ragi tape* from each region, it is found that there is only one type of yeast based on its RFLP pattern. It was only the yeast that comes from Yogyakarta found to contain three types of yeasts. Of the six yeast isolates originating from Yogyakarta *Ragi tape* with four isolates have the same genetic diversity with the yeast of *Ragi tape* from Medan and Cianjur, and two isolates are different from the others.

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