Mycoscience 38: 441–445, 1997

Short Communication

Identification and characterization of yeast isolated from Indonesian fermented food

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Accepted for publication 10 October 1997

Yeast strains with amylolytic activity were isolated from cassava *tapé* and its precursor, *ragi*. They were divided into two groups based on their characteristics: group 1, possessing high amylolytic activity and low ethanol yield; and group 2, possessing low amylolytic activity and high ethanol yield. The major strains of the group 1 were identified as *Endomyces fibuliger*, and those of group 2 were identified as *Pichia anomala*. Based on 18S rDNA analysis, an isolate from *ragi* that had a high amylolytic activity was thought to be an undescribed species that was related to the basidiomycetous genera.

Key Words——18S rDNA analysis; Indonesian fermented food; *ragi*; *tapé*; yeast identification.

Tapé is a traditional Indonesian fermented food made from starch and *ragi*, the microbial starter mixture. Both *tapé* and *ragi* are considered as good sources of amylolytic microbes. *Mucor*, *Rhizopus*, and *Aspergillus* species are thought to be major microorganisms for starch degradation in *tapé* production (Ko, 1972). Several yeast isolates with amylolytic activity were identified as *Endomyces fibuliger* Lindner (synonym: *Saccharomycopsis fibuliger* (Lindner) Klöcker or *Endomycopsis fibuliger* (Lindner) Dekker) and *Pichia anomala* (Hansen) Kurtzman (synonym: *Hansenula anomala*(Hansen) H. & P. Sydow) (Steinklaus, 1983). Itoh et al. (1994) showed that the major amylolytic activity in *tapé* production was due to *E. fibuliger*. To investigate the distribution of yeast species and population diversity we collected 15 samples of cassava *tapé* and 12 samples of *ragi*, and screened for isolates possessing amylolytic activity.

Cassava *tapé* and the starter *ragi* were collected at food markets in Java and Bali. Pre-cultivation was done by shake culture after transferring a loopful of sample into YMPD medium (yeast extract (3 g/L), malt extract (3 g/L), Bacto Peptone (5 g/L), glucose (10 g/L)) at 30°C for 2 d. The isolation medium consisted of agar (20 g/L), Bacto Peptone (5 g/L), glucose (10 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KH_2PO_4 (1 g/L), 0.5% rose bengal solution (0.5 ml/L), 0.2% dichloran solution (1 ml/L), and penicillin (100 U/L). After 2 d of cultivation on the isolation medium at 25°C, single colonies showing different colony-surface and edge characteristics were transferred to agar slants (YMPD medium) and stored at 4°C. Isolates were spotted on agar plates and cultivated for 2 d at 30°C using isolation medium, where the glucose was replaced by soluble starch. The diameter of transparent halo was scored from − (no halo) to +5 (largest halo) after development with KI (0.1 N) − I_2 (0.2 N) solution. To examine amylase excretion activity at cold temperatures, the ratio of halo diameter to the colony diameter (halo-colony ratio) was measured after storage of the agar plates at 4°C for 2 d.

Amylase activity in liquid culture was tested by incubating test isolates (30°C for 4 d) in YMP-starch medium (yeast extract (3 g/L), malt extract (3 g/L), Bacto Peptone (5 g/L), soluble starch (10 g/L)). The culture was centrifugated and the supernatant (1 ml) was added to soluble starch solution (1 ml; 20 g/L). The starch was hydrolyzed for 1 h and 3 h at 37°C and the reaction stopped by adding cold perchlorate (0.2 ml; 35% v/v). The solution was neutralized with KOH (2.5 M) and glucose concentration was determined enzymatically with a food test kit for D-glucose (Boeringer). The ethanol yield of several isolates was determined after growth in shake culture at 30°C for 3 d in YMPD medium containing 150 g/L glucose. Ethanol concentration in the culture supernatant was determined by HPLC with Shodex SH1011 column (Showa Denko, Japan) and RI detector.

Identification methods used were as described (Barnett et al., 1990), and the results were analyzed by use of a yeast identification software (Barnett, 1994). Identification keys used are indicated in Table 3.

DNA extraction and PCR amplification of 18S rDNA

Table 1. Amylolytic activity and ethanol yield of yeast isolates from *ragi*.

Isolate No.	Halo size	Halo-colony ratio	Glucose produced[a] (g/L)		Ethanol yield[b] (g/L)
			1 h	3 h	
R1	+3	3.7		n.t.[c]	5.1
R2	+3	3.3		n.t.	6.4
R3	+4	2.8		n.t.	6.8
R4	+3	2.2	0.16	0.34	40.7
R5	+5	3.5	1.15	2.67	8.2
R6	+3	3.2	1.53	2.76	8.0
R7	+3	3.0		n.t.	10.3
R8	+3	3.2		n.t.	11.2
R9	+3	3.4	1.70	3.23	10.3
R10	+3	2.8		n.t.	4.5
R11	+4	3.0		n.t.	10.6
R12	+3	3.3	1.65	3.30	10.3
R13	+3	3.0		n.t.	10.3
R14	+3	2.0	0.12	0.30	32.5
R15	+3	2.0		n.t.	44.6

a) After incubation of culture supernatant with soluble starch.
b) After fermentation in glucose 150 g/L YMPD medium for 3 d.
c) n.t.: not tested.

Table 2. Amylolytic activity and ethanol yield of yeast isolates from *tapé*.

Isolate No.	Halo size	Halo-colony ratio	Glucose produced[a] (g/L)		Ethanol yield[b] (g/L)
			1 h	3 h	
T1	+3	2.0	0.03	0.09	52.5
T2	+3	3.3	0.07	0.18	36.5
T3	+3	2.7		n.t.[c]	30.4
T4	+3	2.7	0.05	0.12	47.3
T5	+3	3.1	0.14	0.36	40.4
T6	+3	2.2		n.t.	29.8
T7	+3	2.2	0.01	0.02	56.2
T8	+3	2.0		n.t.	39.2
T9	+3	2.0		n.t.	42.9
T10	+3	2.2		n.t.	28.6
T11	+3	2.0	0.13	0.33	54.7
T12	+4	3.5	0.04	0.06	37.9
T13	+3	2.0		n.t.	54.4
T14	+3	2.3	0.13	0.36	54.5
T15	+3	2.0		n.t.	36.5
T16	+3	2.0	0.05	0.12	45.1
T17	+4	1.8		n.t.	n.t.
T18	+4	1.7	0.25	0.43	n.t.
T19	+4	1.9		n.t.	n.t.
T20	+4	1.9		n.t.	n.t.
T21	+4	1.6		n.t.	n.t.
T22	+4	1.8	0.31	0.89	n.t.

a) After incubation of culture supernatant with soluble starch.
b) After fermentation in glucose 150 g/L YMPD medium for 3 d.
c) n.t.: not tested.

were carried out according to Fukatsu and Ishikawa (1996). The fungal body was cultured in YMPD broth, collected by centrifugation and transferred to a mortar. Here it was ground to a fine powder with liquid nitrogen. The DNA was then extracted from the powder. Almost the entire length of 18S rDNA, approximately 1.7 kb in size, was amplified by PCR using primers NS1 and F18SB1. The PCR product was purified by use of a Geneclean II kit (BIO 101 Inc.) and cloned using TA-cloning vector pT7Blue (Novagen) and *E. coli* JM109 competent cell (Takara). Vectors containing the PCR product were subjected to dye-terminator labelled cycle sequencing reactions using 11 sequencing primers (Fukatsu and Ishikawa, 1996), and analyzed by use of a Model 373 DNA sequencer (Applied Biosystems). Determined partial sequences were edited and connected using DNASIS V3.0 (Hitachi Software Engineering). For molecular phylogenetic analysis, DNA databases were searched by

Table 3. Identification data of isolates from *ragi* and *tapé*.

Identification keys	Isolate No., species name																	
	T1	T2	T4	T5	T7	T11	T12	T13	T14	T16	T19	*Pichia anomala*	R5	*Cryptococcus laurentii*	R6	R9	R12	*Endomyces fibuliger*
F1 D-Glucose fermentation	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+D
C2 D-Galactose growth	−	−	+	+	−	+	+	+	+	−	−	+D	+	+	−	−	−	−
C6 D-Xylose growth	+	−	−	+	−	+	+	+	+	−	−	+−	+	+	−	−	−	−
C7 L-Arabinose growth	−	−	−	−	+	D	−	−	+	−	−	−	+	+	−	−	−	−
C8 D-Arabinose growth	−	−	−	+	−	−	+	+	−	−	−	−	+	+	−	−	−	+−
C9 L-Rhamnose growth	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
C10 Sucrose growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+−
C11 Maltose growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C14 Cellobiose growth	+	+	+	+	+	+	+	+	+	+	+	+D	+	+	+	+	+	+
C17 Melibiose growth	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
C18 Lactose growth	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
C19 Raffinose growth	+	+	+	+	+	+	+	+	+	+	+	+−	+	+	+	+	+	+−
C20 Melezitose growth										+	−	+−	+	+	−	−	−	−D
C21 Inulin growth										−	−	−	−	−D	−	−	−	−
C22 Starch growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+−	+	+	+	+
C23 Glycerol growth										+	+	+	+	+−	+	+	+	+
C24 Erythritol growth	+	+	+	+	+	+	+	+	+	+	+	+D	+	+−	−	−	−	+
C26 Xylitol growth	+	+	+	+	+	+	+	+	+	+	+	+D	+	+	−	−	−	−D
C28 D-Glucitol growth	+	+	−	+	+	+	+	+	+	−	+	+	−	+D	−	−	−	+−
C29 D-Mannitol growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+−
C30 Galactitol growth										−	−	−	−	+	−	−	−	−
C35 D-Gluconate growth	+	+	D	D	+	D	+	+	+	+	−	+−	+	+	−	−	−	+D
C36 D-Glucuronate growth										−	−	−	+	+	−	−	−	+
C38 DL-Lactate growth										+	+	+	−	+−	−	−	−	−
C39 Succinate growth	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	+
C40 Citrate growth	+	−	+	+	+	+	+	+	+	+	+	+	−	+−	−	−	−	+−
C44 Butane 2,3 diol growth	+	+	+	+	+	+	+	+	+	+	−	+D	−	−	+	+	+	?
N1 Nitrate growth	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
N4 L-Lysine growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+−	−	−	−	+−
N5 Cadaverine growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+−	−	−	−	+−
N8 Glucosamine growth	+	−	−	−	−	+	−	−	−	−	−	−	−	+−	−	−	−	?
V1 w/o vitamines growth	+	+	+	+	+	+	+	+	+	+	+	+	−	+−	−	−	−	−
0.01% Cycloheximide growth	−	−	−	−	−	−	−	−	−	−	−	−	+	+−	+	+	+	+
0.1% Cycloheximide growth	−	−	−	−	−	−	−	−	−	−	−	−	+	+−	+	+	+	+D

D: Delayed growth (7 d after or later).
Note: T1-T19 were identified as *Pichia anomala*; R5 as *Cryptococcus laurentii*; R6, R9 and R12 as *Endomyces fibuliger*.

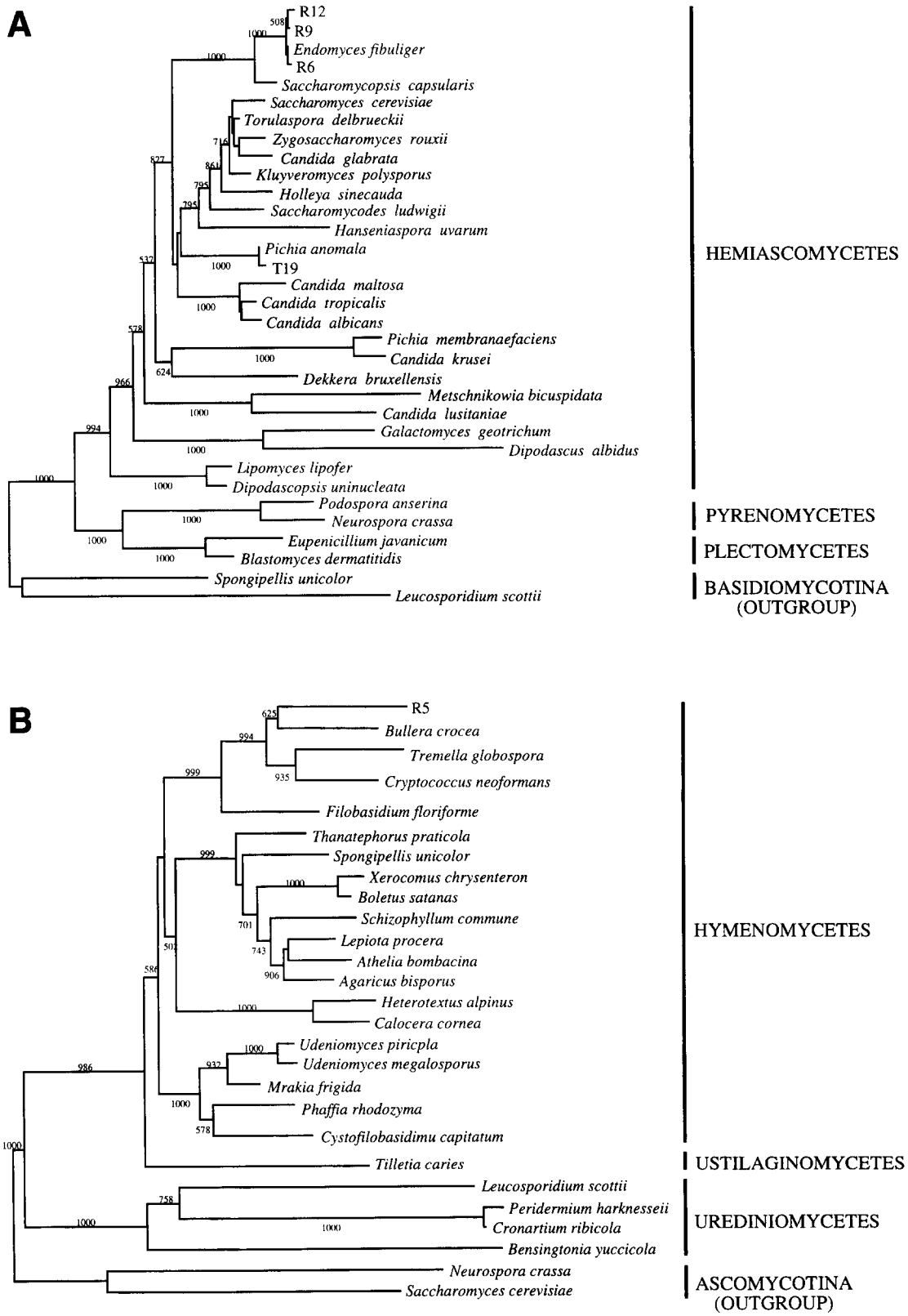

Fig. 1. Molecular phylogenetic position of isolated yeast strains based on 18S rDNA sequences. (A) Phylogenetic position in the Ascomycotina. 1745 aligned nucleotide sites were subjected to analysis. (B) Phylogenetic position in the Basidiomycotina. 1739 aligned nucleotide sites were subjected to analysis. Both are neighbor joining phylogenies constructed by CLUSTAL W. Numbers on the trees show bootstrap support of each clade out of 1,000 replications. Bootstrap values of lower than 50% are not shown. Names of major higher taxa are indicated at right.

use of Entrez Browser and Blast Search on the website of the National Center for Biotechnology Information. Multiple-alignment, neighbor-joining phylogeny construction and bootstrap test were performed by use of CLUSTAL W (Thompson et al., 1994).

From *ragi* samples, yeast strains possessing high amylolytic activity (halo size >3) were isolated. Glucose concentration after incubating with starch solution, which indicated amylolytic activity, is shown in Table 1. Four strains showing a large halo-colony ratio had a high amylolytic activity, giving more than 2.5 g/L of glucose after incubation for 3 h. Two strains (R4 and R14) with a small halo-colony ratio had a low amylolytic activity: less than 0.4 g/L of glucose was produced after incubation for 3 h. Fifteen isolates were examined for ethanol fermentation activity. Twelve strains, which had a halo-colony ratio larger than 2.8, gave a low ethanol yield, i.e., 4–11 g/L ethanol. Three strains (R4, R14 and R15) with halo-colony ratio less than 2.2 gave more than 30 g/L of ethanol.

The amylolytic activity and ethanol yield from 12 *tapé* isolates with a halo size larger than 3 and halo-colony size larger than 2 were examined (Table 2). All strains showed low amylolytic activity but could produce more than 30 g/L of ethanol. No strains similar to the isolates from *ragi* with low ethanol (4-11 g/L) and high amylolytic activity were found.

Eleven isolates from *tapé* and four isolates from *ragi* were subjected to identification tests. Results are shown in Table 3. The *tapé* isolates were all closely related to *P. anomala*. However, all strains failed to match the identification characteristics of *P. anomala* by one or two keys. Three isolates (R6, 9, 12) from *ragi* that had a large halo-colony ratio and reduced ethanol yield were closely related to *E. fibuliger*. However, the growth spectra differed by three keys from *E. fibuliger*, i.e., growth on erythritol, D-glucuronate, and succinate. One isolate from *ragi* (R5), which did not ferment glucose but had a high amylolytic activity, was closely related to *Cryptococcus laurentii* (Kufferath) Skinner, but four identification keys did not match, i.e., growth on melibiose, D-glucitol, galactitol, and succinate.

The phylogenetic position of five isolates (R5, R6, R9, R12 and T19) was examined by molecular phylogenetic analysis based on 18S rDNA sequences. The sequence data were deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers D86910 for R5, D86911 for R6, D86912 for R9, D86913 for R12 and D86914 for T19. When the sequences were subjected to DNA database homology search, R6, R9, R12 and T19 showed a high homology to the sequences of ascomycetous fungi, whereas R5 was homologous to basidiomycetous species. Figure 1 represents the phylogenetic position of the yeast strains based on 18S rDNA sequences. R6, R9, R12 and T19 were in the clade of the Hemiascomycetes in the Ascomycotina. The sequences of R6, R9 and R12 were almost identical to *E. fibuliger*. The sequence of T19 was highly homologous to *P. anomala* (Fig. 1 (A)). In the Basidiomycotina, R5 sequence was in the clade of *Bullera crocea* Buhagiar, *Tremella globospora* Reid and *Cryptococcus neoformans* (Sanfelice) Vuillemin, and this was supported by 99.4% bootstrap value (Fig. 1B). However, R5 was not closely related to any sequence deposited in the DNA databases. An identification test showed that R5 was closely related to *C. laurentii*. A partial sequence of 18S rDNA of *C. laurentii* (574 bp) deposited in DNA databases also showed high similarity, approximately 95%, to that of R5.

These results indicate that T19 is classified as *P. anomala*. Isolates R6, R9 and R12 are likely to be *E. fibuliger*. Isolate R5 is probably an undescribed species related to *Bullera*, *Tremella* and *Cryptococcus*.

The major isolates of *ragi* with high amylolytic activity, which were identified as *E. fibuliger*, were not found in *tapé*, but isolates with high ethanol yield and low amylolytic activity, identified as *P. anomala*, were found in both. It is proposed that during *tapé* production the high amylolytic activity isolates of *ragi* degrade starch and grow on the resultant glucose. The major isolates of *tapé* then rapidly grow on the released glucose to produce ethanol. Previously, we have isolated non-amylolytic yeast species from *tapé*, for example, *Saccharomyces cerevisiae* Hansen (Kuriyama et al., 1985). Such strains are also considered to contribute to ethanol formation in *tapé* production.

The growth spectra of all isolates examined did not match perfectly any known species (Table 3), indicating the variety of strains existing in *tapé* and *ragi*. Thus, it is suggested that *tapé* and *ragi* can be useful sources for screening of amylolytic yeast strains.

Acknowledgement——We thank D.B.Murray for critical reading of this manuscript.

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