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Microflora and Selected Metabolites of Potato Pulp Fermented with an Indonesian Starter *Ragi Tapé*

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Summary

When potato pulp was mixed with Indonesian starter *ragi tapé* and incubated, both lactic acid and ethanol were gradually formed and attained certain concentrations during 2 days of fermentation. Viable counts of fungi in fresh weight matter, yeasts and lactic acid bacteria after fermentation were 10^5, 10^7 and 10^5 CFU/g, respectively. Denaturing gradient gel electrophoresis of the PCR-amplified internal transcribed spacer of 18S–28S rRNA genes detected *Amylomyces rouxii-Rhizopus oryzae*, *Mucor indicus*, *Candida tropicalis* and *Saccharomycopsis fibuligera* and revealed that *Amylomyces rouxii-Rhizopus oryzae* dominated throughout the fermentation period. *Amylomyces rouxii* cannot be discriminated from the lactic acid-accumulating group of *Rhizopus oryzae* because the amplified sequences of these fungi were shown to be identical. Morphological characteristics were then studied for *Rhizopus*-like fungi isolated from fermented potato pulp. Those strains that had produced an enormous number of chlamydospores in the aerial and substrate mycelium were identified as *Amylomyces rouxii*. The microflora of fermented potato pulp was similar to that made from glutinous rice, namely *tapé ketan*.

Key words: Amylomyces rouxii, lactic acid fermentation, *ragi tapé*

Introduction

In Asian countries, dried powders, flat cakes or hard balls are often used as inocula for the production of fermented foods and alcoholic beverages from starchy materials (*1*). These starters contain fungi, yeasts and lactic acid bacteria and are referred to by different names in each area (*2,3*): *ragi tapé* in Indonesia, *look-pang* in Thailand, *chin-hueh* in China, *bubod* in the Philippines, *nuruk* in Korea and *murcha* in Himalayan regions. Lee and Fujio (*4*) have isolated some microorganisms from *banh men*, a starter from Vietnam, and identified the fungal strains as *Amylomyces rouxii*, *Mucor circinelloides*, *Mucor indicus* and *Rhizopus oryzae*, and the yeast strains as *Candida pelliculosa*, *Pichia burtonii*, *Pichia anomala*, *Saccharomyces cerevisiae* and *Saccharomycopsis fibuligera*, based on the morphological and biochemical characteristics. Similar microbial composition has been reported for other fermentation starters (*1,3*).

Conventional Indonesian *tapé* is fermented glutinous rice produced by using *ragi tapé* and consumed as a

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sweet dessert or snack (*5–7*). Among the microorganisms present in *ragi tapé*, the fungus *Amylomyces rouxii* plays a crucial role in *tapé* fermentation. *A. rouxii* degrades starch to glucose, which sustains its growth and that of some yeasts and bacteria, and simultaneously synthesizes both lactic acid and ethanol. Development of the characteristic and acceptable flavor of *tapé* further requires the growth of certain yeasts in addition to *A. rouxii*.

Raw materials for *tapé* are glutinous rice, cassava and other crops containing a high amount of starch. Potatoes (*Solanum tuberosum*) are rarely used in Indonesia (*2*). Through the research into the efficient utilization of agricultural by-products, we suspected that potato pulp could be substituted for an ingredient of *tapé*. Potato pulp containing starch, cellulose, hemicellulose and pectic substances emerges in the starch industry as the residue extracted from potato tubers (*8*). The pulp is crumbled and usually composted or used as cattle feed after being dried or ensiled. Recently, *R. oryzae*, which is taxonomically related to *A. rouxii*, has been used as an alternative inoculant for ensiling potato pulp (*9*). The final goal of our research project is to convert potato pulp to palatable foodstuff by microbial fermentation. In the present study, microflora of *tapé* made with potato pulp has been compared to *tapé ketan*, the conventional *tapé* made with glutinous rice.

Materials and Methods

Fermentation tests

Potato pulp (dry matter 20.8 %) was provided by a local plant that manufactures starch from potato tubers. A commercial *ragi tapé* (Na Kok Liong, Solo, Indonesia) was purchased from a market in Yogjakarta, Indonesia in September 2002. The potato pulp (400 g of fresh matter) was cooled to room temperature after being autoclaved at 121 °C for 15 min, then it was mixed with 4 g of *ragi tapé* and put on Petri dishes in the amount of 50 g for fermentation at 27 °C for 4 days. Portions of the samples were weighed and extracted with saline solution (0.85 % NaCl) to analyze organic acids and ethanol using high-performance liquid chromatography with the method described elsewhere (*10*). Glutinous rice was autoclaved at 121 °C for 15 min after soaking at room temperature for 2 h and used instead of potato pulp as a reference.

Enumeration of microorganisms

Five grams of the fermented pulp were suspended in 30 mL of sterilized saline solution (0.85 % NaCl) and homogenized by repeating three times in a Multi-bead Shocker (Yasui Kikai Co., Osaka, Japan) for 60 s with intervals of 1 min. The suspension was diluted serially and spread on the defined media for the enumeration of microorganisms. The media and incubation conditions were as follows: MRS agar containing 10 μg/L of cycloheximide for lactic acid bacteria, 30 °C for 2 days in an anaerobic chamber; potato dextrose agar for yeasts, 27 °C for 2 days; malt extract agar for fungi, 30 °C for 1 day. Yeasts and fungi were discriminated based on their appearance.

DNA extraction

The suspension containing 5 g of the fermented potato pulp in 30 mL of TE (10 mM Tris, 1 mM EDTA, pH=8.0) was supplemented with lysozyme (0.1 mg/mL), labiase (0.1 mg/mL) and *N*-acetylmuramidase (30 μg/mL) and was incubated at 37 °C for 30 min. After centrifugation, DNA was extracted and purified from the precipitate by UltraClean Soil DNA Isolation Mega Prep Kit (Mo Bio Laboratories, Inc., Carlsbad, CA).

PCR amplification

The entire region of the internal transcribed spacer between 18S and 28S rRNA genes (ITS) was first amplified by using the primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) targeted to 28S and 18S rRNA genes, respectively (*11*). The product was passed through a MicroSpin S-300 HR Column (Amersham Bioscience) to remove residual primers. A second PCR was conducted to attach a GC--clamp by using the primers ITS5-GC (5′-CGCCCGCCGGGCGCGGCGGGCGGGGCGGGGGCACGGGGGAAGTAAAAGTCGTAACAAGG-3′) and ITS6 (5′-CGTTCTTCATCGATGCGAGA-3′). The reaction mixture contained 1 μL of the product of the first PCR, 10 pM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 2.5 U AmpliTaq Gold (PE Applied Biosystems). These components were mixed in 1 × AmpliTaq Gold Buffer (PE Applied Biosystems) to obtain a final volume of 50 μL. Initial denaturation was done at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 70 °C for 1 min and elongation at 72 °C for 1 min, with the final elongation at 72 °C for 5 min. The annealing temperature was reduced by 1 °C per cycle from 70 to 65 °C and adjusted to 65 °C after the fifth cycle. After each PCR reaction, 10 μL was electrophoresed on 1.5 % agarose gel to check the amplification. The products in the remainder of the reaction mixture (40 μL) were concentrated to 10 μL by ethanol precipitation and used for the following experiments.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was performed with a DCode Universal Mutation Detection System (Bio-Rad Laboratories). A PCR sample was applied directly to 8 % polyacrylamide gels in a 1 × TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH=8.3) with a denaturing gradient ranging from 20 to 50 %. One hundred percent of denaturant corresponds to 7 M urea and 40 % formamide. The gradient gel was cast using the Gradient Delivery System, Model 475 (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was run at a constant voltage of 65 V and at 60 °C. After the electrophoresis was run for 16 h, the gel was stained with SYBR Green I (Molecular Probes, Eugene, OR) at 10 000 × dilution in 1 × TAE for 30 min and photographed on a UV illuminator. DGGE bands were excised from the denaturing gels, re-amplified with primers ITS5 and ITS6, as described above, and sequenced by each primer. A search of the GenBank database was conducted using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Results and Discussion

The data in this study are representative results of experiments performed in three replicates.

The metabolic production of lactic acid and ethanol is shown in Fig. 1 along with the development of the microbial population. Lactic acid was formed in accordance with pH reduction and was accompanied by the production of ethanol. Concentrations of lactic acid and ethanol decreased after 2 and 3 days of incubation, respectively. The viable count of all microorganisms increased within one day of fermentation except for lactic acid bacteria. The replacement of glutinous rice with potato pulp stimulated the growth of yeasts and lactic acid bacteria, resulting in high amounts of ethanol and lactic acid. In both cases, the population of fungi seemed to be much lower than that of yeasts, but the former values may have been underestimated. A single colony appearing as a fungus is not derived from a single cell such as yeasts because of mycelial growth (*12*). The contribution of fungi to the fermentation may be more significant than expected based on the evaluation of the colony-forming units (CFU) (*6*). However, this assumption cannot be proved by the classical microbiological approach.

Fig. 2 shows DGGE profiles of DNA harvested from fermented potato pulp and glutinous rice. The diagrams include five minor bands that are hardly visible in the photographs. When individual bands appearing in DGGE profiles were sequenced, four of seven bands were affiliated to *Amylomyces rouxii-Rhizopus oryzae*, indicating their dominance throughout the fermentation period. One of three major bands was derived from *Mucor indicus*. It is unknown why multiple bands originated from *A. rouxii-R. oryzae* on DGGE gels even though these bands had identical sequences. We suppose that the appearance of these minor bands is the result of non-uniformed dissociation depending on their nucleotide sequences. When large amounts of a single PCR product are loaded on DGGE gels, minor bands may emerge in addition to the major band. Among yeasts, *Candida tropicalis* disappeared just after the fermentation, and *Saccharomycopsis fibuligera* emerged on the third day. Further growth of *S. fibuligera* in the fermented glutinous rice, as judged from an additional band (h) in Fig. 2, is related to vigorous production of ethanol after 3 days (Fig. 1).

In DGGE gels, the yeast DNA band was faint, whereas *A. rouxii-R. oryzae* DNA was clearly detected, in contrast to the population detected with the plating method (Fig. 1). This result indicates that, in terms of DNA, *A. rouxii* dominated in the culture. This discrepancy might be due to the fact that yeasts, unicellular organisms, can easily dominate in the plate-counting method, whereas the fungi will develop a single colony from multiple cells. Thus, the method we established here will be useful to identify mixed populations of fungi in traditional fermented foods in Asia.

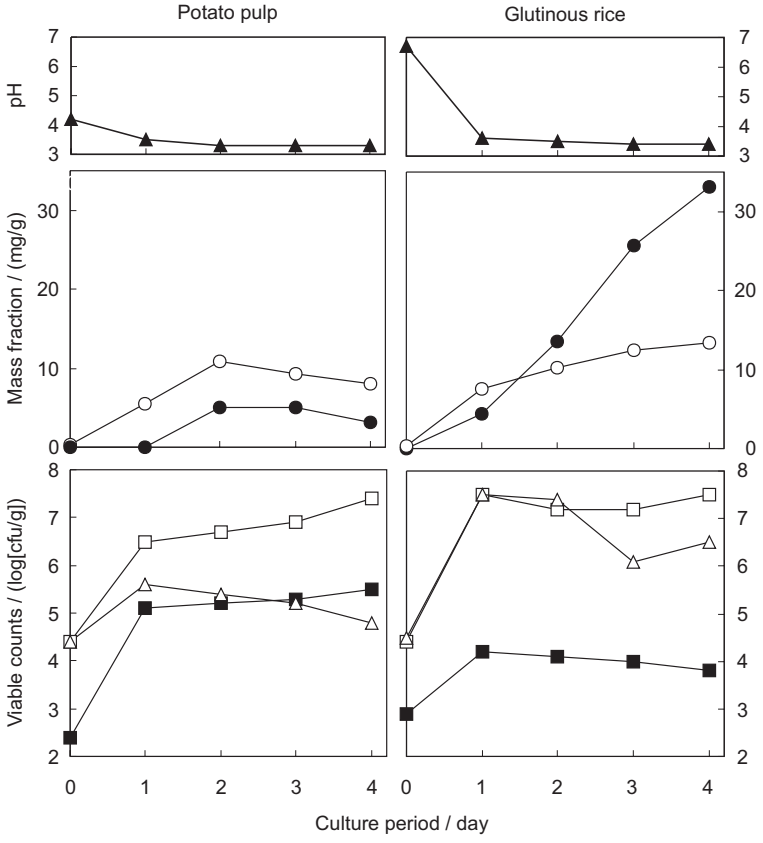

Fig. 1. Biochemical and microbiological parameters monitored during the fermentation of potato pulp and glutinous rice
Potato pulp and glutinous rice were mixed with the starter *ragi tapé* and incubated at 27 °C for 4 days. After the extraction with saline solution (0.85 % NaCl), pH and metabolites were analyzed. Microorganisms in the fermented products were enumerated on the defined media
Symbols: ▲ pH, ○ lactic acid, ● ethanol, ■ fungi, □ yeasts, △ lactic acid bacteria

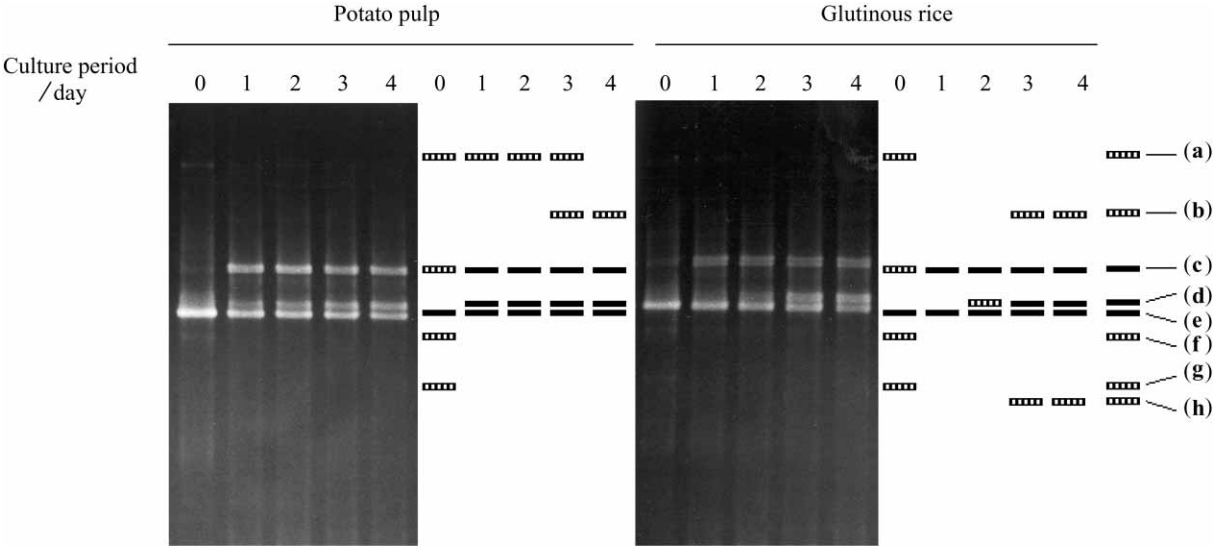

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) profiles of the PCR-amplified ITS1 region of the DNA extracted from fermented potato pulp and glutinous rice
DGGE analysis was conducted for the fermented potato pulp and glutinous rice used in Fig. 1.
Shaded column in the diagram indicates the band invisible in the corresponding photograph. Each band was excised, re-amplified, sequenced and affiliated as follows: (a), (d), (e) and (f), *Amylomyces rouxii-Rhizopus oryzae*; (b) and (h), *Saccharomycopsis fibuligera*; (c), *Mucor indicus*; (g), *Candida tropicalis*

Bacterial communities of fermented foods (*13,14*) and compost (*15*) have been assessed by DGGE for the V3 region of rDNA. DGGE analysis of the 16S–26S rDNA spacer region from bacteria has been useful for discriminating closely related species (*16*) or individual strains in the same species (*17*). For eukaryotic organisms, a limited number of DGGE based studies was conducted using partially amplified 18S rDNA (*18*) and 28S rDNA (*19,20*) but not the ITS region.

Amylomyces is a monotypic genus containing the single variable species *A. rouxii*, which is closely related to *R. oryzae*, as identifiable from the formation of rhizoids, stolons and black-pigmented sporangia (*12*). A distinct morphological characteristic of *A. rouxii* is the enormous number of chlamydospores produced in the aerial and substrate mycelium. Recently, *R. oryzae* strains have been classified into two groups which principally accumulate either lactic acid or fumaric acid in a medium containing relatively high amounts of carbon sources. However, the ITS1 sequence cannot discriminate among *A. rouxii* and lactic acid-accumulating strains of *R. oryzae* because these sequences are identical (*11*). We isolated twenty colonies of fungi from the fermented potato pulp, which were identified as *Mucor indicus* and *A. rouxii* based on morphological characteristics. The appearance of *R. oryzae* was not shown. This has led to the conclusion that *A. rouxii* dominated in the culture of the fermented potato pulp.

From the DGGE pattern (Fig. 2), *A. rouxii* was also found to dominate in the fermented rice, *tapé ketan*, indicating that potato pulp is unlikely to stimulate the growth of undesirable fungi existing in *ragi tapé*. In Indonesia, peeled, diced and steamed cassava tubers are alternatively used for the production of *tapé ketella*. Potato pulp fermented by *ragi tapé* may be consumed as other *tapé* products.

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Mikroflora i odabrani metaboliti krumpirove pulpe fermentirane s dodatkom indonezijske starter kulture *ragi tapé*

Sažetak

Kada se krumpirova pulpa pomiješa s indonezijskom starter kulturom *ragi tapé* i inkubira, postupno se stvaraju mliječna kiselina i etanol koji nakon dva dana fermentacije dostižu određene koncentracije. Nakon fermentacije utvrđeno je u svježoj tvari 10^5 CFU/g gljivica, 10^7 CFU/g kvasca i 10^5 CFU/g bakterija mliječne kiseline. Denaturirajućom gradijentnom gel-elektroforezom PCR-amplificiranih i transkribiranih internih razdjelnih zona (spacer region) iz 18S–28S rDNA gena utvrđeni su *Amylomyces rouxii-Rhizopus oryzae*, *Mucor indicus*, *Candida tropicalis* i *Saccharomycopsis fibuligera*, a ujedno je ustanovljeno da su *Amylomyces rouxii-Rhizopus oryzae* prevladavali tijekom fermentacije. *Amylomyces rouxii* ne može se razlikovati od *Rhizopus oryzae*, koja proizvodi mliječnu kiselinu, jer su amplificirane sekvencije tih gljivica identične. Stoga su proučavane morfološke karakteristike vrsti *Rhizopus* slične gljivice izolirane iz fermentirane krumpirove pulpe. Oni sojevi koji su proizveli golem broj klamidospora u okolišu, i u miceliju supstrata, utvrđeni su kao *Amylomyces rouxii*. Mikroflora fermentirane pulpe krumpira bila je slična kao kod ljepljive riže (*tapé ketan*).