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Note

Comparison of *Amylomyces rouxii* and *Rhizopus oryzae* in Lactic Acid Fermentation of Potato Pulp

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***Amylomyces rouxii*, the filamentous fungus widely used in the production of Asian fermented foods, is closely related to certain strains of *Rhizopus oryzae* secreting lactic acid. Among seven strains of *A. rouxii*, CBS 438.76^T most vigorously produced both lactic acid and ethanol from glucose, starch, and pectin in liquid media. When this strain was grown on apple peels and successively mixed with potato pulp, the concentration of lactic acid produced was lower than that produced by *Rhizopus oryzae* NBRC 4707. However, the growth of *A. rouxii* CBS 438.76^T acidified the pulp to less than pH 4, the level found in conventional silage fermented by lactic acid bacteria. *A. rouxii* may be preferable to *R. oryzae* for recycling potato pulp and other agricultural by-products into food materials because this fungus was being consumed long before written history, which attests to its safety for humans.**

Keywords: pectic substances, polygalacturonase, *look-pang*, *ragi*, fermented food

Certain strains of *Rhizopus oryzae* aerobically produce L-lactic acid from various types of complex heterocarbohydrates (Hang, 1989). The characteristics of *Rhizopus oryzae* are of interest to scientists who study the fermentation of potato pulp, a by-product of the starch industry. One strain, IFO (=NBRC) 4707, was selected from among 38 strains (Oda *et al.*, 2002). The source of this strain can be traced to Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, but descriptions of its origin and properties have been lost in the process of transfer and are now unavailable (http://www.ifo.or.jp). Some strains of *R. oryzae* have been isolated from *tempeh*, a traditional fermented food of Indonesia produced from cooked soybeans (Hachmeister & Fung, 1993). All of these strains synthesize malic and fumaric acids and are included exclusively in a group differing from the strains accumulating lactic acid (Oda *et al.*, 2003). Recently, sequences of 18S-28S rRNA spacer regions were shown to be identical to lactic acid-accumulating strains of *R. oryzae* and *Amylomyces rouxii* but not to strains of *R. oryzae* that produce malic and fumaric acids (Abe *et al.*, 2003). *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 (Ellis *et al.*, 1976). A distinct morphological characteristic that *A. rouxii* and *R. oryzae* share is the enormous number of chlamydospores produced in the aerial and substrate mycelium. *A. rouxii* is a major component of starter cultures for traditional fermented foods in Southeast Asia, China, and the Indian subcontinent (Hesseltine *et al.*, 1988). This fungus may have been unintentionally selected as a mutant from *R. oryzae* because it appears unable to survive in nature (Hesseltine, 1983). The fact that *A. rouxii* was consumed long before written history (Cronk *et al.*, 1977) indicates that it is safer for use in food than *R. oryzae*. In the present experiments, lactic acid fermentation of potato pulp by *A. rouxii* was compared with that of *R. oryzae* strain NBRC 4707.

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Materials and Methods

Organisms One gram of Indonesian *ragi* was suspended in 9 ml of sterilized saline solution (0.85% NaCl). The suspension was diluted serially and spread on the potato dextrose agar. After incubation at 30°C for 1 day, six strains of filamentous fungi showing distinct but *Amylomyces*-like colonies, SDM01, SDM18, SDM23, SDM26, SDM28, and SDM34, were isolated. These strains grew well on sucrose or maltose but poorly on glycerol as the principal carbon source and produced abundant chalamydospores with many abortive sporangia. Their 18S–28S rRNA sequences that are effective to differentiate closely related species (Oda *et al.*, 1997) were identical to that of *A. rouxii* isolate 32233 (Accession No. AF115724) and shared 99.8% identity with that of strain CBS 438.76^T, a type of *A. rouxii* (Accession No. AY238888). From these characteristics (Abe *et al.*, 2003; Ellis *et al.*, 1976), all of the six SDM strains were identified as *Amylomyces rouxii*. Strain CBS 438.76^T was originally isolated from *look-pang* made in Thailand (Ellis *et al.*, 1976). *Ragi* and *look-pang*, types of sweet fermented glutinous rice, are used as starter cultures for traditional foods. *Rhizopus oryzae* IFO 4707, obtained from the Institute for Fermentation, Osaka, Japan, is now available from the National Biological Resource Center (Kazusa, Japan), as NBRC 4707.

Culture Fungal cells were grown on potato dextrose agar prepared horizontally in a standing test tube (12×90 mm). The mycelium was taken from the surface of the agar and inoculated in 50 ml of a medium containing 1.0% glucose, soluble starch (Wako Pure Chemical Ind., Ltd., Tokyo) or pectin from citrus fruits (Sigma), 0.67% yeast nitrogen base without amino acids (Difco), and 0.5% casamino acids (Nippon Seiyaku Co., Tokyo) in a 100 ml Erlenmeyer flask. Calcium carbonate (1.25 g) was added to the medium to neutralize the acid produced before cultivation. After cultivation for three days at 25°C with shaking (90 rpm), the culture filtrate was obtained.

For the fermentation of potato pulp, the mycelium was grown for three days on 10 g of apple peel in a Petri dish as a starter culture and successively fermented for seven days after being mixed with 90 g of the sterilized pulp under air-tight conditions as described previously. The fermented pulp (10 g) was mixed with 30 ml of distilled water and centrifuged to obtain the supernatant. Potato pulp (dry matter 20.8%) was donated by a local processing plant that was manufacturing starch from potato tubers.

Organic acids and ethanol were determined using a high-performance liquid chromatograph (LC-10AVP, Shimadzu, Kyoto) with an RI-detector (Shimadzu). The analytical conditions were as follows: column, Shodex Ionpak KC-811 (Showa Denko Co., Ltd., Tokyo); column temperature, 40°C; mobile phase, 0.1% phosphoric acid; and flow rate, 1.0 ml/min.

Enzyme assays The crude enzyme was extracted from the fermented pulp by an extraction buffer (50 mM malic acid, 50 mM NaCl, 100 mM NaOH, 2 mM $CaCl_2$, pH 5.8). Polygalacturonase was assayed by measuring the reducing sugar released from polygalacturonic acid as described elsewhere (Saito *et al.*, 2003). Commercial kits supplied by Megazyme International Ireland Co., Ltd. (Wicklow, Ireland) were used for the assays of cellulase, α-amylase, and glucoamylase. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar equivalent to galacturonic acid, glucose, or *p*-nitrophenol per min under the assay conditions.

Statistical analysis The data obtained from three independent experiments were statistically analyzed using the *t*-test.

Results

Organic acids and ethanol produced by seven strains of *A. rouxii* in a liquid medium were determined (Table 1). All of the strains synthesized lactic acid as an organic acid and ethanol as found in *R. oryzae* NBRC 4707 and some other strains (Oda *et al.*, 2003). Strains *A. rouxii* CBS 438.76^T produced much more lactic acid and ethanol than the other six strains of *A. rouxii* in all three media. Concentrations of lactic acid in the media containing pectin or soluble starch were significantly less than that containing glucose ($p<0.01$). Pectin and soluble starch may not be degraded by CBS 438.76^T as completely as glucose is.

Potato pulp, an agricultural by-product in the starch industry, contains starch, cellulose, hemicellulose, and pectic substances (Mayer & Hillebrandt, 1997). Its fermentation by *R. oryzae* is governed by the degradation of pectic substances that bind independent cells in the potato tuber (Saito *et al.*, 2003). When the fungal cells were grown on fruit peels containing high amounts of pectin, polygalacturonase activity increased enough to enhance the formation of lactic acid and ethanol in potato pulp (Saito *et al.*, 2003). Similar experiments were conducted for *A. rouxii* CBS 438.76^T as described in Materials and Methods. The concentrations of lactic acid and ethanol shown in Fig. 1 cannot be compared directly with those shown in Table 1 because fungal cells were grown with shaking in a liquid medium supplemented with calcium carbonate to neutralize the acid produced. A decreased pH in potato pulp seemed to reduce

Table 1. Production of lactic acid and ethanol by the seven strains of *Amylomyces rouxii*.

Carbon source	Strain	Amount (mg/ml)*	
		Lactic acid	Ethanol
Glucose	SDM01	0.13±0.12	0.80±0.63
	SDM18	0.41±0.07	2.51±0.62
	SDM23	0.25±0.01	1.15±0.57
	SDM26	0.27±0.11	0.93±0.06
	SDM28	0.20±0.01	0.96±0.08
	SDM34	0.16±0.14	1.38±0.14
	CBS 438.76^T	4.64±0.22	2.16±0.40
Soluble starch	SDM01	0.29±0.04	0.83±0.22
	SDM18	0.48±0.50	0.91±0.38
	SDM23	0.43±0.19	0.91±0.76
	SDM26	0.21±0.05	0.33±0.19
	SDM28	0.16±0.05	0.69±0.24
	SDM34	1.27±0.34	1.72±0.08
	CBS 438.76^T	3.09±0.15	1.83±0.22
Pectin	SDM01	0.40±0.08	0.93±0.59
	SDM18	0.20±0.08	0.96±0.86
	SDM23	0.42±0.07	0.69±0.34
	SDM26	0.19±0.14	0.54±0.40
	SDM28	0.20±0.09	0.81±0.16
	SDM34	0.33±0.20	0.46±0.21
	CBS 438.76^T	3.06±0.63	1.83±0.35

* Mean±SD (n=3).

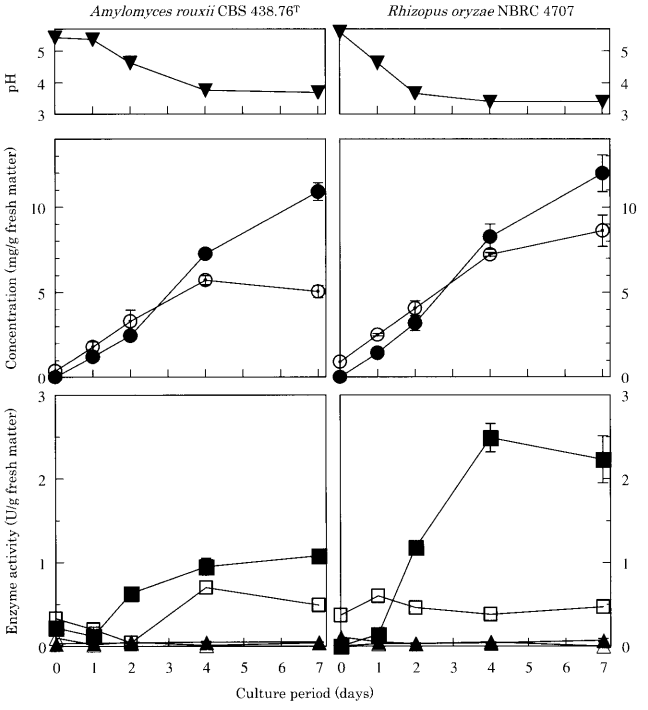

Fig. 1. Production of metabolites and enzymes in potato pulp fermented by *Amylomyces rouxii* CBS 438.76^T and *Rhizopus oryzae* NBRC 4707. Inverted solid triangles, pH; open circles, lactic acid; solid circles, ethanol; solid squares, polygalacturonase; open squares, cellulase; open triangles, α-amylase; solid triangles, glucoamylase.

further the formation of lactic acid after four days of incubation, and pyruvic acid was converted to ethanol. The concentration of lactic acid produced by *A. rouxii* CBS 438.76^T was significantly lower than that produced by *R. oryzae* NBRC 4707 ($p<0.01$), corresponding with the polygalacturonase activity. It is still unknown whether limited growth, suppressed enzyme productivity or inactivation of enzyme accounts for lower activity of polygalacturonase. However, the growth of *A. rouxii* CBS 438.76^T acidified the pulp to less than pH 4, the level found in conventional silage fermented by lactic acid bacteria (Seale, 1986).

Discussion

Traditional foods made in Asia are fermented by a mixed-culture inoculum of filamentous fungi, yeasts, and lactic acid bacteria (Hesseltine, 1983). During the fermentation process, the microorganisms simultaneously convert starchy substrates to alcohol or organic acids. These starter cultures are referred to as *ragi* in Indonesia and Malaysia, *look-pang* in Thailand, *murcha* in Himalayan regions, and *Chin-yueh* in China and Taiwan (Holzapfel, 2002). Indonesian *ragi* consists of rice powder with certain microorganisms and is used for making *tapé ketan*, a traditional sticky rice dessert (Siebenhandl *et al.*, 2001). Abe *et al.* (2003) have developed a detection method for the group including lactic acid-accumulating strains of *R. oryzae* and *A. rouxii* by touchdown PCR using specific primers targeted to ITS regions. SDM strains were arbitrarily isolated from commercial samples of *ragi* and were identified as *A. rouxii*. Differences in metabolite productivity in strain CBS 438.76^T and the SDM strains are unknown but may be explained by their original types and storage conditions.

Rhizopus species are found in *ragi* as well as in *A. rouxii* and are used for brewing alcoholic beverages (Hesseltine, 1991). However, *R. oryzae* occasionally causes the human disease mucormycosis (Ribes *et al.*, 2000) and is a possible pathogen in some industrial crops (Phytopathological Society of Japan, 2000). *A. rouxii* has been consumed by Asian people in fermented foods, including viable fungal cells, as described above, and there has been no report of detrimental effects on animals and plants. These observations indicate that *A. rouxii* may be preferable to *R. oryzae* for use in fermenting potato pulp and other agricultural by-products as food materials.

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