Anales Inst. Biol. Univ. Nac. Autón. México, Ser. Bot. 60(1): 1-7, 30-XI-1990

YEASTS IN AMYLOLYTIC FOOD STARTERS

CLIFFORD W. HESSELTINE*
CLETUS P. KURTZMAN*

RESUMEN

En muchas regiones de Asia se utilizan inóculos secos para iniciar la fermentación de alimentos hechos con arroz y cazabe. Dichos inóculos consisten en cultivos mezclados de levaduras, bacterias y mohos. En un estudio de casi 100 cepas de levaduras aisladas de ragi, murcha, levadura china y bubod, se encontró que las especies predominantes fueron *Saccharomycopsis fibuligera* y, en menor grado, *S. malanga*. Cuando se utilizaron cepas de levaduras seleccionadas junto con varias bacterias obtenidas de estos inóculos iniciadores, la fermentación lao-chao del arroz no fue satisfactoria. Sin embargo cuando se utilizó *S. fibuligera* junto con un moho (*Mucor indicus*) aislado de bubod, sí se obtuvo un alimento fermentado satisfactorio.

Palabras clave: Ragi, levadura china, bubod, murcha, alimentos fermentados.

ABSTRACT

In many areas of Asia, dry starters are used for food fermentations of rice and cassava. Starters consist of mixed cultures of yeast, bacteria and molds. Based upon a study of nearly 100 yeast strains from ragi, murcha, Chinese yeast and bubod, the predominant yeasts were *Saccharomycopsis fibuligera* and, to a lesser extent, *S. malanga*. When selected yeast strains were used with various bacteria from these starters, the lao-chao rice fermentation was not satisfactory. However, when a mold (*Mucor indicus*) isolated from bubod was used along with *S. fibuligera*, a satisfactory food was produced.

Key words: Ragi, Chinese yeast, bubod, murcha, fermented foods.

INTRODUCTION

Starter cultures based on starch from cereals are used in many areas of Asia to initiate food fermentations. The starters or "ferments" are a long-standing tradition that can be traced to ancient times and are still widely used throughout the Orient today. Calmette (1892) was the first to report the presence of several wild yeast species in starters used in Indochina to produce alcohol; they were accompanied by the molds *Amylomyces*, *Mucor* and *Aspergillus* and about 30 different bacteria. The most abundant yeast was similar to *Saccharomyces pastorianus*. According to Went and

* Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

Prinsen-Geerligs (1896), *Monilia javanica* (= *Pichia anomala*) and *S. cerevisiae* were the principal yeasts of ragi in Indonesia. *Saccharomyces* sp. was reported from Chinese yeast (Neuville, 1902) and from bakhar (Ray, 1906), the latter an Indian name for Chinese yeast. Saito (1904) demonstrated the presence of yeast in Shiro-koji from Taiwan, and in a second paper (1908) described the yeast as having 2-spored asci and not forming films in liquid media. This species fermented glucose, galactose, sucrose, maltose and raffinose and produced as much as 5.2% v/v ethanol. Also present was a yeast described as *Mycoderma* sp.

Saito (1913) described a new yeast from a sample of Chinese yeast and demonstrated that the species produced alcohol. This species was described as *Endomyces linderi* (= *Saccharomycopsis fibuligera*). *Saccharomycopsis fibuligera* is typically found growing on cereal products and, therefore, it is not surprising to find it in ragi-like products. Will (1913) likewise described a new species of yeast from "levura anamite" that is used in the amyloprocess. This was *Saccharomyces anamensis* which is today considered to be *S. cerevisiae*.

Takahashi (1915) made the first extensive study of yeasts in Chinese yeast used for making "Shaoshing-chu" rice wine in China. He found *Mucor* to be present and also isolated and described 7 types of *Saccharomyces shaoshing* and 4 types of *Zygosaccharomyces shaoshing.* Neither yeast species is included in modern taxonomic treatments. An especially important paper is the report by Yamazaki (1932) on the yeast flora in Chinese yeast starter cakes. Twenty plates are devoted to pictures of the different size and shape of yeast cakes based upon the 201 samples collected from 17 different Chinese locations. Dwidjoseputro and Wolf (1970) reported the yeasts in ragi to consist of *Candida parapsilosis, C. melinii, C. lactosa, Hansenula subpelliculosa, H. anomala* and *H. malanga*. Some of these strains were later reidentified (Kurtzman *et al.*, 1974).

The tape fermentation depends upon the use of ragi as the inoculum. Djien (1972) demonstrated that use of a mixture of *Amylomyces rouxii* and *S. fibuligera* gave a good fermentation product. The original cultures used by Djien had been isolated from ragi and were grown in rice to produce the experimental inoculum. When *S. fibuligera* was used to inoculate sterile, moist rice, only some white growth developed on the rice kernels. However, when *Amylomyces* and *S. fibuligera* were combined as inoculum, good tape was made. Djien suggested that pure mixed cultures of the two microorganisms could be used as commercial inoculum, and that this mixture can be dehydrated and will remain active at room temperature for at least 5 months. Suprianto *et al.* (1989) also examined the tape fermentation but reported the active microorganisms to be *Rhizopus* sp., *Saccharomycopsis* sp. and *Streptococcus* sp.

The properties of glucoamylase from a ragi isolate of *S. fibuligera* were studied by Kato *et al.* (1976). The purified enzyme released the β-form of glucose during starch hydrolysis and had a high activity toward maltodextrins.

Lin *et al.* (1974) isolated 5 yeasts in starters used in the Shao-hsing wine manufacture in Taiwan. The inoculum, Chiu-yau, comes typically in the form of ragi-like balls used as starters. All the yeasts were believed to be *Saccharomyces cerevisiae,* although there were some slight differences in taxonomic characteristics. Yeasts in

murcha starter cakes of India were studied by Batra and Millner (1974). They found *Hansenula anomala* var. *schneggii*, now considered a synonym of *Pichia anomala*. This yeast was isolated from all samples of murcha but uses soluble starch only after prolonged incubation; therefore, it probably used the sugars produced from the starch by molds.

Hadisepoetro *et al.* (1979) reported that yeast counts in three ragi starters were $0.5\text{-}1.4 \times 10^7$. Eight of the 9 yeasts were *Candida* spp. and 7 of these produced an abundance of true mycelia. These 7 isolates were non-ascosporic, formed a thick pellicle and sediment in broth, assimilated KNO_3, grew in a vitamin-free medium and had an optimum growth temperature of 28-37°C.

Saono and Basuki (1978) reported 13 species of *Candida* from ragi and other traditional fermented foods of Indonesia. Of the 80 yeast strains tested, only 22 were able to utilize starch. Strains of the same species may be active while others are inactive. Twenty of the 22 strains came from ragi. Yeasts isolated from ragi tended to lack lipolytic activity and all strains were inactive proteolytically. In a second paper, the same authors (1979) investigated the amounts of amylolytic enzymes produced. Molds from ragi could utilize glucose and starch as carbon sources for ethanol and biomass production but the yeasts utilized glucose better than starch. Yeasts produced much less reducing sugars in the starch medium, did not change the pH, and produced little biomass.

Amylomyces rouxii and *Pichia burtonii* were studied as a mixed culture to determine the biochemical changes that occurred in starchy substrates during tape fermentations (Cronk *et al.*, 1977). The mixed culture reduced the total solids 50% in 192 h and reduced the starch to 18% of the original in 48 h. With the mixed culture fermentation, the reducing sugar rose from 1% to 16-17% between 36 and 48 h and alcohol was 8% v/v at 144 h; the pH of the mixture was 4.1, but the mold alone reduced the pH to 4.0 in 48 h. In a second paper, Cronk *et al.* (1979) looked at the higher alcohols (fusel oils) produced during the tape fermentation. Isobutanol and isoamyl alcohols were formed, but the mold alone would produce almost as much of these as the mixed culture. In the mixed culture, 72 mg/l fusel oil was formed in 32 h and 558 mg/l at 192 h. When *P. anomala* and *P. subpelliculosa* were used with *Amylomyces*, ethyl acetate accumulated at concentrations ranging from 145-199 mg/l at 36 h to 354-369 mg/l at 192 h.

From our own experience, and from the work of others, we have had the distinct impression that *Saccharomycopsis fibuligera* represents the dominant starch-degrading yeast in amylolytic food starters. In earlier studies, we isolated yeasts from ragi, bubod, murcha and Chinese yeast. Total yeast counts in these products ranged from 2×10^3 - 6.1×10^8 viable cells per g (Hesseltine *et al.*, 1988). In the present work, we address the question of whether *S. fibuligera* is the primary amylolytic yeast species present in these food starters. We also examined the type of fermented rice product obtained following inoculation with selected yeasts, bacteria, and a combination of yeast and *Mucor indicus*, a mold frequently associated with Asian rice fermentations.

TABLE 1
DISTRIBUTION OF YEAST SPECIES FROM AMYLOLYTIC FOOD STARTERS.

Type of Starter	*Frequency of representative isolates*		
	S. fibuligera	*S. malanga*	*Other species*[a]
Bubod	13	0	8
Chinese yeast	9	5	4
Murcha	17	0	9
Ragi	17	2	15

[a]These isolates were tentatively identified as species of *Saccharomyces*, *Pichia* and *Candida*.

TABLE 2
EFFECT OF BACTERIA, YEAST, AND MOLD COMBINATIONS ON THE LAO-CHAO FERMENTATION

Species and NRRL No.	Final pH*	Appearance of Rice				
		Growth	Color Change	Odor	Taste	Liquefaction
S. faecalis						
B-14617	5.48	— —	— —	perfume-like	cooked-rice	— —
B-14618	5.50	— —	— —	perfume-like	cooked-rice	— —
P. pentosaceus						
B-14719	5.95	— —	— —	perfume-like	cooked-rice	— —
P-14620	6.00	— —	— —	perfume-like	cooked-rice	— —
P-14622	5.97	— —	— —	perfume-like	cooked-rice	— —
S. fibuligera						
Y-11999	4.70	dry	mycelial, white	fruity, alcoholic	chalky	— —
Y-12612	4.35	dry	mycelial, white	fruity, alcoholic	chalky	— —
Y-12711	4.28	moist	— —	fruity, alcoholic	chalky, and sour	— —
S. faecalis and *S. fibuligera*						
B-14617 + Y-12711	4.00	slimy	— —	fruity, alcoholic	yeast-like	±**
P. pentosaceus and *S. fibuligera*						
B-14619 + Y-12711	4.17	slimy	— —	fruity, alcoholic	yeast-like	±**
B-14620 + Y-12711	3.84	moist	— —	fruity, alcoholic	yeast-like	±**
S. fibuligera and *M. indicus*						
Y-12711 + 26212	— —	rice liquified	— —	malt-like	sweet-sour	+

*Uninoculated rice = pH 6.8.
**±, kernels were soft on the outside but firm on the inside.

MATERIALS AND METHODS*

On the basis of colony growth, nearly 100 representative yeast strains were isolated from starter samples obtained from Nepal and India (26), Philippines (7), Indochina (50), and China and Taiwan (17), and each strain was lyophilized for future study when first isolated. The representative strains had the following distribution in the starters: ragi, 34; murcha, 26; Chinese yeast, 11; and bubod, 21. For comparison, *Saccharomycopsis fibuligera* NRRL Y-2388, the type strain isolated from bread, and NRRL Y-7170, another strain of this species isolated from ragi, were included. Also compared was NRRL Y-7175, the type strain of *S. malanga* that had been isolated from Indonesian ragi by Dwidjoseputro and Wolf (1970).

In this study, *S. fibuligera* was separated from other yeasts on the following criteria: presence of hat-shaped ascospores, presence of true hyphae, production of a fruity odor, growth at 37°C and growth on soluble starch. Appearance was also distinctive for many strains. Colonies were dry and dusty and formed horn-like projections made up of many strands of mycelium. The species *S. malanga* was recognized by the same criteria with the exception that it produces a somewhat gamey (wet dog) odor (Kurtzman *et al.*, 1974), and colonies show no aerial projections of mycelium. Starch assimilation and other growth reactions were determined as described by Wickerham (1951).

In an effort to simulate rice fermentation as actually used in production of laochao, various mixtures of yeasts, bacteria, and a mold were tested. The yeasts were: NRRL Y-11999 from ragi, Bali; NRRL Y-12612 from ragi, Indonesia; and NRRL Y-12711 from murcha, Nepal. All three strains were identified as *S. fibuligera*. The bacteria selected were: NRRL B-14617 and NRRL B-14618, *Streptococcus faecalis* from ragi, Bali; NRRL B-14619, NRRL B-14620, and NRRL B-14622, *Pediococcus pentosaceus* from ragi, Indonesia (although these bacteria are from ragi, the same species are used for making lao-chao). The mold used was *Mucor indicus* from bubod.

Each culture was grown in TGY (tryptone glucose yeast) broth for 24 h. Five ml of this growth was mixed with 80 g (dry weight) of long-grain rice which had been soaked in water overnight, drained, autoclaved and cooled in 200 ml beakers. The beakers were covered and incubated at 28°C. Evaluation of the fermentation was made after 48 h. As controls, each bacterium and yeast strain was grown by itself on the rice substrate. The pH was determined after blending the fermented rice in neutralized distilled water.

RESULTS AND DISCUSSION

The identification and distribution of yeast species from amylolytic food starters are reported in Table 1. A majority of the isolates were identified as *S. fibuligera*. In view of the widespread use of these starters, the fact that one species predominates seems especially significant. *Saccharomycopsis malanga,* a phenotypically sim-

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

ilar species that also produces amylase, was found in Chinese yeast and ragi, but not in bubod and murcha. The significance of this observation is not presently known but may be reflective of the geographical distribution of the species. The remaining yeasts isolated from these starters represent a mixture of species that predominate in the genera *Saccharomyces, Pichia* and *Candida*. In view of the diversity of these species and their apparent low numbers, we assume that they have no significant role in the fermentation of starchy food products.

Results of rice fermentations employing various combinations of yeasts, bacteria, and a mold are presented in Table 2. A typical lao-chao fermentation gives a sticky product with a sweet-sour taste and a fruity flavor. Although partially liquified, individual rice kernels are still separate. When the rice was inoculated with *S. fibuligera*, white mycelial growth covered the surfaces of kernels, but no liquefaction occurred. Bacteria alone caused no liquefaction, color change, or other evidence of growth. Combinations of yeast and bacteria also failed to give typical lao-chao. However, as demonstrated in Table 2, when *Mucor indicus* was used with *S. fibuligera*, a suitable product was formed. More details will be published in a subsequent paper that documents the mold-yeast interaction in this fermentation.

LITERATURE CITED

BATRA, L. R. and P. D. MILLNER. 1974. Some Asian fermented foods and beverages and associated fungi. *Mycologia 66*:942-950.

CALMETTE, A. 1892. Contribution à l'étude des ferments de l'amidon. La levure chinoise. *Ann. Inst. Pasteur 6*:604-620.

CRONK, T. C., L. R. MATTICK, K. H. STEINKRAUS, and L. R. HACKLER. 1979. Production of higher alcohols *during Indonesia tape ketan fermentation. Appl. Environ. Microbiol. 37*:892-896.

CRONK, T. C., K. H. STEINKRAUS, L. R. HACKLER, and L. R. MATTICK. 1977. Indonesian tape-ketan fermentation. *Appl. Environ. Microbiol. 33*:1067-1073.

DJIEN, K. S. 1972. Tape fermentation. *Appl. Microbiol. 23*:976-978.

DWIDJOSEPUTRO, D. and F. T. WOLF. 1970. Microbiological studies of Indonesian fermented foodstuffs. *Mycopathol. Mycol. Appl. 41*:211-222.

HADISEPOETRO, E. S. S., N. TAKADA, and Y. OSHIMA. 1979. Microflora in ragi and usar. *J. Ferment. Technol. 57*:251-259.

HESSELTINE, C. W., R. ROGERS, and F. G. WINARNO. 1988. Microbiological studies on amylolytic oriental fermentation starters. *Mycopathologia 101*:141-155.

KATO, K., K. KUSWANTO, I. BANNO, and T. HARADA. 1976. Identification of *Endomycopsis fibuligera* isolated from ragi in Indonesia and properties of its crystalline glucoamylase. *J. Ferment. Technol. 54*:831-837.

KURTZMAN, C. P., R. F. VESONDER, and M. J. SMILEY. 1974. Formation of extracellular 3-D-hydroxypalmitic acid by *Saccharomycopsis malanga. Mycologia 66*:580-587.

LIN, C. F., C. C. CHANG, and H. IIZUKA. 1974. Taxonomic studies on the yeasts used in Shao-Hsing wine manufacture. *J. Ferment. Technol. 52*:196-200.

NEUVILLE, H. 1902. Les ferments industriels d'extrême-orient. Ency. Sci. d. aide-Mémoire publiée sous la direction de M. Leaute 1-192. Paris (*Centralb. Bakteriol., 2. Abth. 10*:130-131).

RAY, P. C. 1906. On the Hindu method of manufacturing spirit from rice, and its scientific explanation. *J. Asiat. Soc. Bengal 2*:129-142.

SAITO, K. 1904. Eine neue Art der "Chinesischen Hefe." *Centralb. Bakteriol., 2. Abth. 13*:153-161.

SAITO, K. 1908. Note on some Formosan fermentation organisms. *Bot. Mag. (Tokyo) 22*:4-13.

SAITO, K. 1913. Ein neuer *Endomyces (Endomyces linderi). Zeitschr. f. Gärungsphysiol. 2. 3*:151-153.

SAONO, S. and T. BASUKI, 1978. The amylolytic, lipclytic, and proteolytic activities of yeasts and mycelial molds from ragi and some Indonesia traditional femented foods. *Ann. Bogor. 6*:207-219.

SAONO, S. and. T. BASUKI. 1979. Amylolytic and ethanol producing activities of some mold and yeast strains. *Ann. Bogor. 7*:11-19.

SUPRIANTO, R. OHBA, T. KOGA, and S. UEOA. 1989. Liquefaction of glutinous rice and aroma formation in *tapé* preparation by *ragi. J. Ferment. Bioengin. 67*:249-252.

TAKAHASHI, T. 1915. Observations on the microorganisms of the mash of "Shaoshing-chu" and "Chu-ya." *J. Coll. Agric. Imp. Univ. Tokyo. 5*:199-266.

WENT, F.A.C. and H. C. PRINSEN-GEERLIGS. 1896. Beobachtungen über die Hefearten und zuckerbilden-den Pilz der Arackfabrikation. *Verh. Kon. Ned. Akad. Wetensch., Afd. Natuurh., Tweede Sect. II 4*:3-31.

WICKERHAM, L. J. 1951. Taxonomy of yeasts. *U.S.D.A. Techn. Bull. 1029*:1-56.

WILL, H. 1913, *Saccharomyces anamensis*, die Hefe des neueren Amyloverfahrens. *Centralb. Bakteriol. 2 Abth. 39*:26-53.

YAMAZAKI, M. 1932. Studies on the "Chinese-yeast-cakes" - "Chu" 1. Some characteristics of Chinese-yeast cakes. *Bull Utsonomiya Agric.* Coll. 1:1-24.