Taxonomical examination and characterization of a methanol-utilizing yeast

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The morphological, cultural, and physiological characteristics are described of a yeast, LI70, which uses methanol as its source of energy and carbon; these characteristics have made it possible to identify the strain as *Candida boidinii* Ramirez. The identification was confirmed by a DNA-DNA genetic homology of 99.43% with the type strain of *C. boidinii*. Strain LI70 is not pathogenic.

INTRODUCTION

Yeasts capable of utilizing methanol are in nature particularly abundant in soil and water rich in decomposing woody residues, fruit and other vegetable products, tree exudates and bark, and fermentation liquids (Ramirez, 1953; Santa Maria, 1958; Ogata et al., 1970; Asthana, Humphrey and Moritz, 1971; Sahm and Wagner, 1972; Hazeu, de Bruyn and Bos, 1972; Oki et al., 1972; Levine and Cooney, 1973; Volfová and Pilát, 1974; Kato et al., 1974; Cooney, Makiguchi and Montgomery, 1975; Tezuka et al., 1975; Henninger and Windisch, 1975). We screened samples of these materials by direct isolation and enrichment cultures and were thus able to confirm that methanol-utilizing yeasts can be basically ascribed to the following genera: *Candida*, *Torulopsis*, *Kloeckera*, *Hansenula*, and *Pichia*. The degree of utilization of methanol was independent of the genus to which the strains belong.

Earlier papers described the production on a pilot-plant scale and some data on the chemical composition of a yeast, strain LI 70, with a high protein content, which grows on methanol (Cardini, Di Fiore and Zotti, 1975; Cardini, Cavalli and Calcagno, 1976). The present paper reports the results of the taxonomical

examination and characterization and pathogenicity testing of this yeast strain, isolated from chips of larch bark.

MATERIALS AND METHODS

The following yeast strains were used: LI 70 SIR; *Candida boidinii* CBS 2428 (type strain); CBS 2429, CBS 3092, CBS5325, CBS 5777, CBS 6056; *Torulopsis pinus* CBS 970; *Hansenula philodendra* CBS 6075; *Hansenula nonfermentans* CBS 5764. In addition, a series of yeasts obtained from collections and having well-defined responses to individual physiological tests were used as controls.

The taxonomical study was conducted according to methods and criteria described by Lodder (1971), Barnett (1968), Barnett and Pankhurst (1974) and Campbell (1974), and with special tests that are pointed out. A recently introduced method "Api 20 Candida" (Grillot et al., 1973; Grillot, 1974) was also used. Durham's tubes and other methods were used for the fermentation tests, bearing in mind the considerations presented by Skinner and Fletcher (1960), Scheda and Yarrow (1966), and van der Walt (1971).

The vitamin requirement for growth was determined after four successive transfers in liquid medium. The capacity to use amino acids as the carbon source in the absence of other nitrogen sources was assessed by turbidimetry according to the method of Hedrick and Dupont (1968). The LI 70 strain's temperature requirements for growth (Minimum, Optimum and Maximum) were determined after a week in solid medium using a polythermostat with sixteen scalar slots (cf. Guicciardi et al., 1968). The growth at 37 C (two weeks) of all the strains examined was determined in liquid medium. Lipolytic activity was determined with the method suggested by Fryer, Reiter and Lawrence (1967).

Taking into account the suggestions of De Ley and Tijtgat (1970), the membrane filter technique proposed by Denhardt (1966) and already applied to yeasts by Bicknell and Douglas (1970), was used to determine the degree of genetic homology between the LI 70 yeast strain and the reference species. The labeled DNA of the type strain CBS 2428 was extracted from cells cultivated for 30 hours at 25 C with agitation in broth containing 5% glucose and 0.5% yeast extract, supplemented with ^{14}C uracil and ^{14}C adenine (50 μCi/litre, each). The procedure of Martini, Phaff and Douglass (1972) was followed for the extraction and purification of the DNA using acetone powders of the cells. A Sartorius SM 11306 membrane filter was used for the DNA fixation. The hybridization temperature was maintained at 58 C in view of the Tm of the strains studied. The radioactivity of the filter disks was measured with a Phillips LSA 420 liquid scintillation counter.

For checking whether the LI 70 yeast strain has any pathological activity, a variety of tests was carried out employing suitable experimental inoculation

procedures (Ciferri, 1958; Segretain, Drouhet and Mariat, 1974) in mice (CD_1 Swiss, weighing about 18 g), rats (CD Sprague Dawley, weighing about 120 g), and rabbits (New Zealand, weighing about 2 kg). Each treatment group was compared to a group of control animals. The animals were assigned to the groups randomly. They were housed at a temperature of 22–24 C and a relative humidity of 68–72%, fed a standard diet ad libitum, and controlled for a six-week period (deaths, general state of health, weight gain). They were then sacrificed and a macroscopic anatomo-pathological examination was carried out.

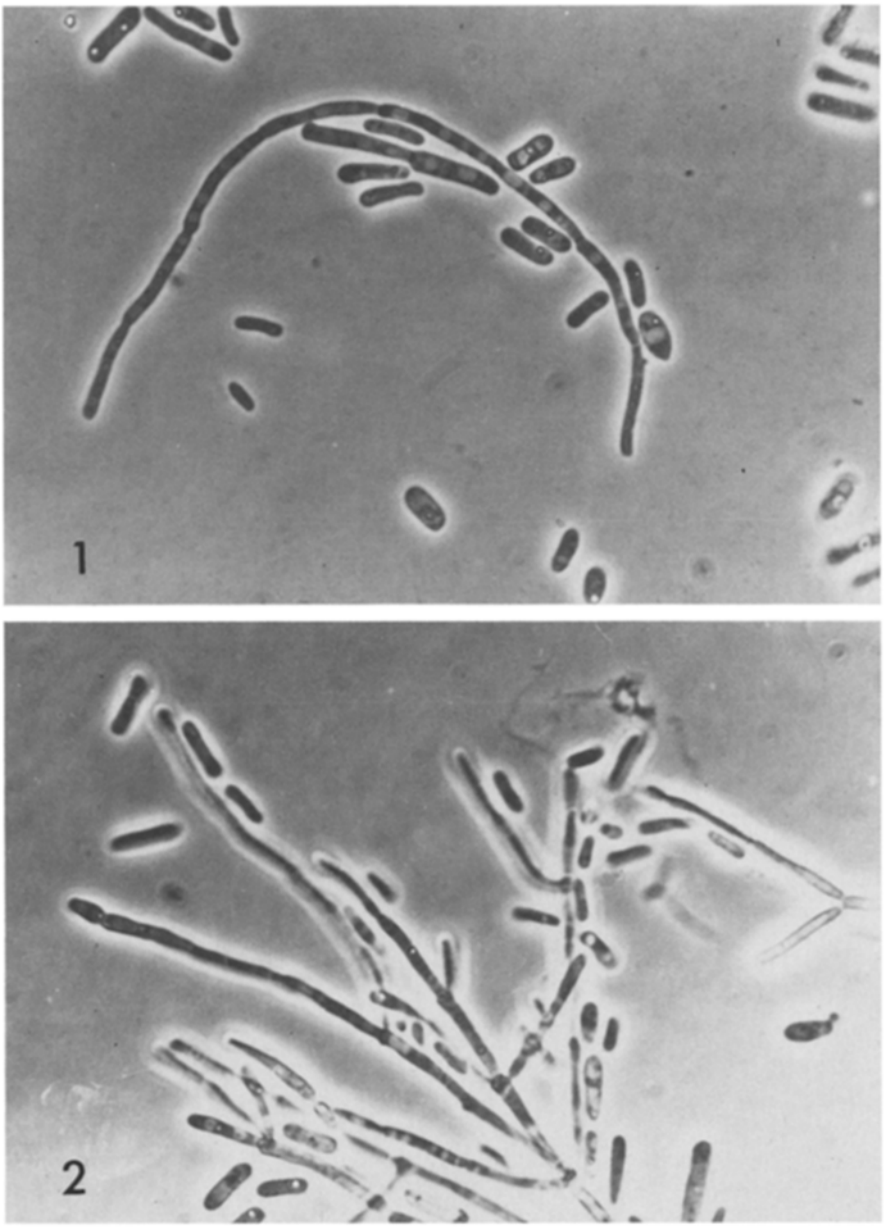
The animal studies were performed with dilutions of a suspension of live cells in physiological saline containing 40 million cells/ml (S). The cells were obtained by culture in broth (Wickerham's yeast – nitrogen base + 3% glucose) for 36 hours at 28 C and were washed three times. In a first series of tests, each group contained 15 male animals and the following routes of administration were used: intravenous in the mouse (0.5 ml S 1/20) and the rabbit (2.5 ml S), intraperitoneal in the rat (1.5 ml S 1/10), oral in the mouse (beverage ad libitum of S 1/200) and in the rat (1.5 ml S 1/10 by probe), intracutaneous in the rat (0.3 ml S by distribution at two points after depilation) and inhalation of aerosol in the rat (S 1/20 for 15 minutes, repeated after 15 days). In all the subsequent tests each group contained 10 male and 10 female animals. In one test, three intravenous inoculations were made at three-day intervals in the mouse (0.5 ml S 1/20 × 3) and in the rabbit (2.5 ml S × 3). In additional tests it was attempted to favor the onset of experimental infection by treating the animal beforehand with an antibiotic or administering mucin together with the microorganism. For the first procedure tetracycline was administered to the mouse and rabbit orally with a probe (100 mg/kg/day for six consecutive days) and the yeast suspension was inoculated intravenously (0.5 ml S 1/20 in the mouse; 2.5 ml S in the rabbit) on the eighth day after the beginning of the antibiotic treatment; for the second procedure the cell suspension S diluted in a 10% solution of gastric mucin was administered intraperitoneally to the mouse (0.5 ml S 1/20) and rat (1.5 ml S 1/10).

RESULTS AND DISCUSSION

The LI 70 yeast strain has the following characteristics:

Growth in glucose–yeast extract–peptone broth: after two–three days at 25 C the cells occur single or paired, elongated, ovals or cylindrical, sometimes slightly curved (1.6–3 × 5–12 μm) (Fig. 1). Chains of cells or a pseudomycelium can also be present. A faint, dry, rising pellicle is formed.

Growth on glucose–yeast extract–peptone agar and on malt agar: after four weeks at 25 C the thin, light cream-colored growth is only faintly wrinkled. Extracellular pigment does not form.



Figs. 1 and 2. Phase-contrast photomicrographs of *Candida boidinii* strain LI 70. Single cells, chains, and pseudomycelium. 1200 ×.

Dalmau plate cultures on corn meal agar: an abundant pseudomycelium (Fig. 2) is produced; it consists of branched chains and pseudohyphae on which blastospores can be seen.

Ascospore formation was never observed.

Fermentation: positive, although generally weak, for glucose, fructose, and mannose (gas evolution is more evident in Durham's tubes with 5% glucose when a large amount of inoculum is used); negative for galactose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, starch, and methyl-α-D-glucoside.

Assimilation of carbon compounds: positive for D-glucose, D-fructose, D-mannose, D-xylose, L-arabinose (weak), D-ribose, methanol, ethanol, glycerol, erythritol, ribitol, D-mannitol, D-glucitol, acetate, lactate, succinate, and malate; negative for D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, starch, glycogen, D-arabinose, L-rhamnose, propanol, isopropanol, galactitol, citrate, inositol, methyl-α-D-glucoside, salicin, amygdalin, esculin, and arbutin.

Utilization of nitrogenous compounds: positive for ammonium sulfate, nitrate, nitrite, and ethylamine.

Utilization of amino acids as the C and N source: positive for proline, glutamic acid, aspartic acid, and alanine and weakly positive for arginine; negative for cysteine, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine.

Growth in a vitamin-free medium: negative, with a biotin requirement.

Growth under osmotic pressure: positive for 5% NaCl and 10% glucose; negative for 10% NaCl and 50% glucose.

Liquefaction of gelatin: negative.

Lipolytic activity: positive.

Production of extracellular starch: negative.

Resistance to cycloheximide (100 μg/ml): positive.

Temperature requirements for growth: Min. about 4 C, Opt. 24–28 C, Max. 32–33 C.

On the basis of the above findings, the LI 70 yeast strain was identified as *Candida boidinii;* this species was first described, although incompletely and without full confirmation, by Ramirez (1953) and subsequently by Santa Maria (1958) as *Candida olivarium (C. boidinii* CBS 3092) and by Yokotsuka and Goto (1955) as *Candida koshuensis*, later identified by Grinbergs and Yarrow (1970) as *C. boidinii* (CBS 5777).

In addition to the minor morphological differences, the following observations should be stressed: most important, the LI 70 strain showed less fermentation than any of the *C. boidinii* reference strains examined; the L-arabinose assimilation test reported as positive or negative by Lodder (1971), was in our study decidedly positive for the strains CBS 2428, CBS 2429, and CBS 5777, weakly positive for the strains LI 70, CBS 3092, and CBS 5325, and negative

for CBS 6056; the succinate assimilation test, reported as negative by Lodder (1971), was in our study positive for LI 70 and the six *C. boidinii* strains from collections. It should be noted that Sahm and Wagner (1972) and Tezuka et al. (1975) reported a positive succinate assimilation test for two strains identified as *C. boidinii*. The growth test at 37 C was negative for the LI 70 and CBS 2428 strains and positive for the other five.

With the DNA-DNA hybridization technique used in this study, the degree of genetic homology with respect to the type strain *C. boidinii* CBS 2428 was 99.55% for CBS 3092, 99.43% for LI 70, 98.83% for CBS 6056, 97.09% for CBS 5325, 80.07% for CBS 5777, 78.58% for CBS 2429, and 0% for *H. philodendra*. These findings indicate that all of the aforementioned *Candida* strains, including LI 70, are very closely related genetically to the *C. boidinii* Ramirez type strain, confirming the correctness of their taxonomical position. It should be noted that the *H. philodendra* strain, with physiological characteristics similar to those of *C. boidinii* but different morphology, and purposely selected as a reference, did not show an appreciable percentage of reassociation.

The study conducted in laboratory animals with the LI 70 yeast strain to ascertain any pathological effects gave the following results: no deaths occurred in any of the groups of animals; no symptoms attributable to a pathological state were noted; liveweight changes were always within normal limits; the macroscopic anatomo-pathological examination did not reveal any organ lesions or untoward effects.

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