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The Yeast Flora of Some Decaying Mushrooms on Trunks of Living Trees

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ABSTRACT. Several ascomycetous and basidiomycetous yeasts were isolated from rotten mushrooms on the trunks of beech and tamarisk trees. One strain, identified as the novel species *Cryptococcus allantoinivorans*, assimilated allantoin as the sole carbon source. Phylogenetically it belongs to the *C. laurentii* complex, *Papiliotrema bandonii* being the closest relative. Some ascomycetous strains could not be distinguished from *Pichia guillermondii*, but deviated considerably in rDNA sequences. In addition to these species, both decaying mushrooms were inhabited by more common species, *viz*. *Candida albicans*, *C. saitoana*, *Rhodotorula mucilaginosa*, *Trichosporon asahii*, *T. multisporum* and *T. porosum*. The basidiomycetous yeasts, except *R. mucilaginosa*, assimilated some polysaccharides of plant origin.

Several yeast species assimilate a great variety of carbon sources in addition to those used for yeast identification and taxonomy (Middelhoven *et al.* 1985, 1991, 2001, 2004*a*; Middelhoven and Kurtzman 2003). Uric acid is one of these nonconventional substrates (Middelhoven *et al.* 1983). Like other aerobic microorganisms, ascomycetous and a basidiomycetous yeast species degrade uric acid by oxidation to allantoin that, in three successive hydrolytic steps, is converted into 1 mol glyoxylate and 2 mol urea (Middelhoven *et al.* 1983). Surprisingly, most yeast species assimilating uric acid as the sole carbon source failed to grow at the expense of allantoin, in spite of equal energetic value. Middelhoven *et al*. (1985) demonstrated that of 14 ascomycetous and basidiomycetous yeast species, growing on uric acid as the sole carbon source, only *Stephanoascus ciferrii* M.TH. SMITH *et al.* assimilated allantoin. It is wide-spread in nature and is a source of nitrogen for many yeast species. Nevertheless, several attempts to isolate yeasts from soil by enrichment culture on allantoin as the sole carbon source failed. In the present study such yeasts were looked for in another habitat, *viz*. decaying mushrooms. It has been known for a long time that mushrooms contain allantoin (Brunel 1931, 1936). Mushrooms also contain chitin as a constituent of the fungal cell wall. Assimilation of this polysaccharide by yeasts has never been reported. Attempts were made to isolate such yeasts from decaying mushrooms.

The present study was not restricted to isolation of yeasts with characteristic biochemical abilities. In addition, several yeast species present in decaying mushrooms were identified as representatives of well-known species. However, some very rare species and some strains physiologically similar to *Pichia guillermondii* but belonging to undescribed species were also isolated. The basidiomycetous yeasts were tested for growth on several polysaccharides of plant origin.

MATERIALS AND METHODS

Isolation of the strains. The rotting mushrooms sampled were *Hericium erinaceus* (BULL.:FR.)PERS. on the trunk of a beech tree (*Fagus sylvatica* L.) in Wageningen (The Netherlands) September 2000, and *Inonotus tamaricidis* (PAT.) MAIRE on the trunk of a tamarisktree growing near the seaside in Plakias (Crete, Greece) October 2000. Suspensions of the rotten material were plated on YM agar plates (in g/L: glucose 10, peptone 5, yeast extract 3, malt extract 3, agar 20) supplied with 20 mg/L of streptomycin sulfate and chloramphenicol in order to suppress growth of bacteria. Samples of the rotting mushrooms were also diluted 1 : 10 in mineral salts GB medium (Middelhoven *et al.* 1991, 2001) without carbon and nitrogen source supplied with antibiotics *as above*. Cultures of 50 mL in 300-mL Erlenmeyer flasks were incubated on a rotary shaker at 20 °C for 3 d or more. Attempts were made to isolate yeast strains able to grow on allantoin or colloidal chitin as sole carbon source by subcultivation in GB medium supplied with 2 g/L allantoin or chitin.

Yeasts were isolated as described above. Samples of rotting material flowing down the trunks were also treated.

Ascomycetous yeast strains were maintained on YM agar slants, basidiomycetes on PDA (potato dextrose agar; *Difco*) slants at 4 °C.

Identification of the strains. The strains were examined for morphological and physiological properties using standard yeast identification methods (Yarrow 1998; Barnett *et al.* 2000). For identification, the BioloMICS programm provided by Dr. V. Robert (*Centraalbureau voor Schimmelcultures* (CBS), Utrecht, The Netherlands) was used; it is available on internet page: **www.cbs.knaw.nl** . Identification of some *Trichosporon* strains and the *Cryptococcus* strain was confirmed by nuclear base sequencing of the D1D2 and ITS regions of 26S ribosomal DNA (Fell *et al.* 2000; Scorzetti *et al.* 2002).

Assimilation of polysaccharides was tested in GB medium (Middelhoven 2004*a*) containing 40 mmol/L NH_4Cl at pH 6.5, inulin and soluble starch at 5 g/L, dextran (*Sigma*; from *Leuconostoc* sp.), pullulan (*Sigma*, filter-sterilized) and galactomannan (*Sigma*; from locust bean) at 2.5 g/L, xylan (*Sigma*; from birch wood) at 10 g/L; the insoluble fraction was removed by centrifugation. Polygalacturonate and tannic acid were tested in 5 g/L GB medium at pH 5.5. Tests were done in culture tubes with 2 mL culture medium incubated on a rotary shaker at 25 °C for 7 d.

RESULTS AND DISCUSSION

The isolated strains are listed in Table I. Some strains were deposited in the yeast culture collection of the CBS (Utrecht, The Netherlands). Yeasts could easily be isolated from decaying mushrooms by plating a suspension directly on YM agar plates. The stream of rotting material down the fruit body of *H. erinaceus* yielded no yeasts but green molds. When incubated under laboratory conditions in GB medium, when the rotten material was the only source of carbon and nitrogen, yeasts dominated, favored by antibiotics that suppressed growth of bacteria.

Table I. Yeast species isolated from decaying mushrooms

Inoculum	Yeast identified	Strain
	H. erinaceus	
Directly	*Trichosporon porosum* (STAUTZ) MIDDELHOVEN, SCORZETTI et FELL	BS-1
Stream below, *via* GB medium	*Candida albicans* (ROBIN) BERKHOUT	BF-1
	Pichia guillermondii-like	BF-2 (CBS 9454)
Enriched on allantoin	*Cryptococcus allantoinivorans* MIDDELHOVEN	ABB-3 (CBS 9604)
	I. tamaricidis	
Directly	*Candida saitoana* NAKASE et M. SUZUKI	TF-1 and TF-4
	Pichia guillermondii-like	TF-2 (CBS 9453)
Via GB medium	*Kodamaea ohmeri* (ETCHELLS et BELL) YAMADA et al.	TA-1 (CBS 9452)
	Rhodotorula mucilaginosa (JÖRGENSEN)HARRISON	TA-3
	Trichosporon asahii AKAGI ex SUGITA et al.	TA-2
Stream below, *via* GB medium	*Candida saitoana* NAKASE et M. SUZUKI	TAF-2
	Trichosporon multisporum COCHET	TAF-3 (CBS 9201)

C. albicans is a well-known opportunistic pathogen. Strain BF-1 assimilated *n*-hexadecane and grew at 30 °C, but not at 32 °C. This low maximum growth temperature excluded identification as the closely related *C. tropicalis* (CASTELLANI) BERKHOUt and suggested that it is avirulent.

From *I. tamaricidis* three strains of *Candida saitoana* were isolated. These strains grew weakly on propane-1,2-diol. This excludes identification as *C. famata* (HARRISON) MEYER et YARROW, which is the anamorph of *Debaryomyces hansenii* (ZOPF) LODDER et KREGER-VAN RIJ. Other physiologically very similar species are *C. globosa* KOMAGATA et NAKASE and *C. pseudoglobosa* SUZUKI et NAKASE (Suzuki and Nakase 1993). Following the instructions of the latter authors, strains TF-1, TF-4 and TAF-2 were identified as *C. saitoana* as they grew at 35 °C, assimilated glycerone ('dihydroxyacetone') albeit weakly and L-arabinose, but not D-arabinose.

Three strains initially identified as *Pichia guillermondii* were isolated from both mushrooms. These strains deviated from strains of this species by forming creeping pellicles. The physiological data (Table II) agree with the description given by Kurtzman (1998) and Barnett *et al*. (2000), but sequencing of 26S rDNA

Table II. Physiological characteristics of *Pichia guillermondii*-like yeast strains[a]

Test	*Pichia guillermondii* (Barnett *et al*. 2000)	*Kodamaea ohmeri* TA-1 (CBS 9452)	Undescribed species BF-2 (CBS 9454)	Undescribed species TF-2 (CBS 9453)
Carbon source				
L-Sorbose	V	+	–	–
D-Glucosamine	+D	+	+D	+D
D-Ribose	+D	–	–	–
D-Xylose	+	W	+	+
L-Arabinose	V	–	–	–
L-Rhamnose	V	–	–	–
Methyl α-D-glucoside	V	+	+	+
Cellobiose	V	+	+	+
Melibiose	V	–	–	–
Raffinose	+D	+	–	–
Melezitose	V	–	+	+
Soluble starch	–W	–	–	–
D-Glucitol	V	+	+	+
D-Mannitol	V	+	+	+
2-Keto-D-gluconate	+	+	+	+
D-Gluconate	V	–	+D	–
DL-Lactate	V	–	–	–
Fermentation				
D-Glucose	+D	+	–D	+
D-Galactose	V	D	–	+
Sucrose	+D	+	–	–
Raffinose	V	+	–	–
Nitrogen source				
Nitrite	V	–	–	–
Ethanamine	+	+	+	+
L-Lysine	+	+	+	+
Cadaverine	+	+	+	+
Miscellaneous				
10 ppm cycloheximide	+D	+	D	+
Urease	–	–	–	–
DBB stain[b]	–	–	–	–
Growth at (EC) 30	V	+	+	+
32	V	+	–	–
37	V	+	–	–
40	V	+	–	–
42	V	–	–	–

[a](+) – growth within 8 d, D – growth after 8–20 d, (–) – no growth after 20 d, V – variable results, W – weak growth response. All strains of *P. guillermondii* studied by Barnett *et al*. (2000) and the 3 strains isolated from rotting mushrooms had many characteristics in common: all assimilated D-glucose, D-galactose, sucrose, maltose, α,α´-trehalose, glycerol, citrate and *n*-hexadecane as the sole carbon source, and ethanamine, L-lysine and cadaverine as the sole nitrogen source, and tolerated 10 % NaCl, but none assimilated lactose, erythritol, *myo*-inositol, 5-keto-D-gluconate, D-glucuronate, D-galacturonic acid, methanol, butane-2,3-diol, D-glucarate, D-galactonate, nitrate and D-tryptophan.

[b]DBB – '*p*-diazobenzoyl biocytin'.

showed that they belong to different species. All three strains required biotin for rapid growth but strains BF-2 and TA-1 grew slowly when supplied with thiamin as vitamin. The three strains differed from each other in some characteristics. Strain TA-1 fermented glucose, galactose and sucrose, grew at 40 °C but not at 42 °C and failed to grow on D-xylose. Slide cultures on Yeast morphology agar (*Difco*) showed mainly ovate

budding cells with some pseudomycelium under the cover slip. By sequencing of 26S rDNA it was identified as *Kodamaea ohmeri* (ETCHELLS et BELL) YAMADA et al., syn. *Pichia ohmeri* (ETCHELLS et BELL) KREGER-VAN RIJ. Like *P. guillermondii* it is a teleomorph of *Candida guillermondii* (CASTELLANI) LANGERON et GUERRA. Strain BF-2 fermented glucose slowly and weakly, produced abundant branched pseudomycelium with constrictions and blastoconidia in slide cultures on yeast morphology agar, but ovate budding cells only under the cover slip. Strain BF-2 grew at 30 °C but not at 32 °C. Strain TF-2 fermented glucose and galactose, but not sucrose. Slide cultures on yeast morphology agar showed only ovate budding cells, but septate-branched mycelium was observed in slide cultures on maize meal agar. It grew at 30 °C, but not at 32 °C. Sequencing of 26S rDNA showed that strains BF-2 and TF-2 were not conspecific with any other described yeast species. For this reason both strains were deposited in the *CBS Culture Collection* where they received accession numbers CBS 9454 and 9453, respectively. The sequences were deposited in GenBank: CBS 9453 26S rRNA gene large subunit AY498861, ITS1 and ITS2 AY498862; CBS 9454 26S rDNA gene large subunit AY498863, ITS1 and ITS2 AY498864. Introduction of novel species to accommodate these strains should be postponed until more strains with identical rDNA sequences will have been isolated.

In addition to ascomycetes, some basidiomycetous yeast species were isolated.

Rhodotorula mucilaginosa was isolated from *I. tamaricidis*. Distinction from *R. glutinis* (FRESENIUS) HARRISON is difficult, both species being variable in many characteristics. Strain TA-3 was identified as *R. mucilaginosa* as it assimilated nitrite as the sole nitrogen source and showed multilateral budding.

Both samples yielded one or two species of *Trichosporon* BEHREND, a genus characterized by assimilation of many phenolic compounds (Middelhoven *et al.* 2001). Many species are able to assimilate polysaccharides such as the hemicelluloses, xylan and pectin (Middelhoven 2004*a*). *T. asahii* is a pathogen (most strains kept in culture collections had been isolated from clinical specimens); it has also been isolated from snail excrements (Middelhoven 2002) and from rotten *Euphorbia canariensis* (strain CBS 8520). Strain TA-2 deviated from many other strains of *T. asahii* by its failure to grow on D-arabinose, salicin and propane-1,2-diol. Its identity with *T. asahii* was confirmed by base sequencing of rDNA.

T. porosum (syn. *Apiotrichum porosum* STAUTZ) is very common in the soil in The Netherlands (Middelhoven *et al.* 2001) and was also enriched from soil beneath *Sequoia sempervirens* (coast redwood) in California, USA (Middelhoven 2003). Strain *T. porosum* BS-1 perfectly fitted the standard description.

T. multisporum strain TAF-3 had to be identified by base sequencing of ribosomal DNA as it deviated in many characteristics from the type strain CBS 2495, isolated from rat droppings (Cochet 1940). Guého *et al.* (1998) treated *T. multisporum* as a synonym of *T. laibachii* (WINDISCH) GUÉHO et M.TH. SMITH. The species was reinstalled by Middelhoven *et al.* (2001) as strain CBS 2495 could be distinguished from *T. laibachii* by some base pairs in the D1D2 region of ribosomal DNA and by some physiological characteristics. Strain TAF-3 is the second strain that could be identified with certainty as *T. multisporum*, but deviated considerably from the type strain CBS 2495 by its failure to assimilate L-sorbose, L-rhamnose, ribitol, xylitol, L-arabinitol, D-glucitol, D-mannitol, D-gluconolactone and D-galacturonic acid and by tolerating 0.1 % cycloheximide.

Table III. Assimilation of some polysaccharides by basidiomycetous yeasts from rotting mushrooms

Polysaccharide	*C. allantoinivorans* ABB-3 (CBS 9604^T)	*R. mucilaginosa* TA-3	*T. asahii* TA-2	*T. multisporum* TAF-3 (CBS 9201)	*T. porosum* BS-1
Inulin	–	–	–	–	–
Starch	–	–	+	+	+
Pullulan	–	–	+	–	–
Dextran	–	–	–	–	+
Xylan	+	–	–	–	+
Polygalacturonate	+	–	W[a]	+	+
Galactomannan	–	–	+	+	+
Tannic acid	–	–	–	–	+

[a]Weak growth response.

Enrichment cultures on allantoin, inoculated with decaying mushrooms, resulted in only one strain, ABB-3, from *H. erinaceus* which was identified by traditional methods as *Cryptococcus laurentii* (KUFFERATH) SKINNER. Strain ABB-3 assimilated allantoin as the sole carbon and nitrogen source, but not uric

acid. This unique biochemical feature necessitated confirmation of the identification by nuclear base sequencing of rDNA D1D2 and ITS regions. This revealed that strain ABB-3 (CBS 9604) represents a novel species, to be named *Cryptococcus allantoinivorans* and to be published elsewhere (Middelhoven 2004*b*). It is a close relative of *Papiliotrema bandonii* (Sampaio *et al*. 2002), of *Cryptococcus nemorosus* and *C. perniciosus* (Golubev *et al*. 2003) and of *C. flavescens* and *C. aureus* (Takashima *et al*. 2003). All these species failed to grow on allantoin and uric acid as the sole carbon sources (Middelhoven 2004*b*).

No yeasts could be isolated from enrichment cultures on colloidal chitin but the basidiomycetous yeasts isolated from rotten mushrooms assimilated several other polysaccharides of plant origin (Table III), just as many saprotrophic *Trichosporon* species did (Middelhoven 2004*a*). The ascomycetes displayed little activity toward these polymers (*not shown here*).

Thanks are due to Dr. G. Scorzetti (*University of Miami*, USA) for identification of *Trichosporon* and *Cryptococcus* species by nuclear base sequencing of the 26S subunit of rDNA and to Dr. V. Robert (CBS Utrecht) for sequencing the three *Pichia guilliermondii*-like strains.

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