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Trichosporon veenhuisii sp. nov., an alkane-assimilating anamorphic basidiomycetous yeast

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A morphological and physiological description of an alkane-assimilating anamorphic basidiomycetous yeast species, named *Trichosporon veenhuisii*, is presented. The ability to assimilate several aliphatic and aromatic compounds as sole source of carbon and energy is reported. The phylogenetic position within the genus, based on nuclear base sequencing of the D1/D2 region of the large subunit of rDNA, is discussed. The type strain is CBS 7136^T.

Keywords: alkane utilization, basidiomycetes, taxonomy, *Trichosporon veenhuisii* sp. nov., yeasts

INTRODUCTION

Trichosporon is a genus of anamorphic yeasts (Basidiomyceta, Hymenomycetes, Tremelloidae) with a distinct morphological characteristic of true mycelia that disarticulate to form arthrospores. The genus, in general, consists of soil- and water-associated species, although some species are causative agents of diseases in man (Kwon-Chung & Bennett, 1992). With the exception of *Trichosporon pullulans*, the genus is monophyletic as viewed by small-subunit rDNA (SSU; Sugita & Nakase, 1998) and large-subunit rDNA (LSU; Fell *et al*., 1995, 1999) sequence analyses. Guého *et al*. (1998), in a review of the genus, accepted 19 species. Sugita & Nakase (1998), based on an SSU analysis of the type strains, reported that the species could be divided into three major groups of distinct species.

The standard technique for species identification of yeasts is based on phenotypic characteristics; however, a major difficulty in obtaining accurate identifications of basidiomycetous yeasts is the different within-species strain responses to many of these tests, such as utilization of carbon and nitrogen compounds. Consequently, strains can be mis-identified and the existence of genetically distinct species can be overlooked. One such strain was Mint-301, which Middelhoven *et al*. (1985) isolated from buffalo dung and deposited at the Centraalbureau voor Schimmelcultures (CBS) as CBS 7136^T. That strain had the unique characteristic of the ability to utilize n-hexadecane, a characteristic which, among species of *Trichosporon*, had only been found in *Trichosporon aquatile* Hedrick *et* Dupont (Barnett *et al*., 1983). However, CBS 7136^T did not correspond by taxonomic characteristics to *T. aquatile*. Therefore the strain was temporarily placed in the taxon with the most similar taxonomic characters: *Trichosporon cutaneum* (de Beurmann *et al*.) Ota.

Sequence analysis in our study of the D1/D2 region of the LSU demonstrated that CBS 7136^T was distinct from *T. aquatile*, *T. cutaneum* and all other members of the genus. Therefore, in the following presentation, we propose the new species, *Trichosporon veenhuisii*, based on morphological and physiological characteristics as well as phylogenetic relationships to other species of *Trichosporon*.

Abbreviations: LSU, large-subunit rDNA; SSU, small-subunit rDNA.

The GenBank accession number for the *Trichosporon veenhuisii* sequence of the D1/D2 region of the large-subunit rDNA is AF105400.

METHODS

Isolation of the strain. CBS 7136^T was isolated (Middelhoven *et al*., 1985) from an enrichment culture (30 °C) with DL-2-hydroxypropylamine as the sole source of carbon, energy and nitrogen. The inoculum was buffalo dung from Minturno (Province of Campania, Italy). The strain was grown pure and maintained on YM agar.

Characterization of the strain. Strain CBS 7136^T was examined for morphological and physiological properties with standard yeast identification methods (van der Walt & Yarrow, 1984). Utilization of carbon and nitrogen sources in liquid Difco YNB and YCB was examined at 25 °C on a rotary shaker at a speed of 100 r.p.m. Utilization of nitrite was confirmed by the auxanographic technique. Assimilation of n-hexadecane was shown by the slant culture

Table 1. Strains examined

Strain	CBS no.	GenBank no.
Mrakia frigida	5270^T	AF075463
Trichosporon aquatile	5973^T	AF075520
Trichosporon asahii	2479^T	AF105393
Trichosporon asteroides	2481^T	AF075513
Trichosporon brassicae	6382^T	AF075521
Trichosporon cutaneum	2466^T	AF075483
Trichosporon coremiiforme	2482^T	AF139983
Trichosporon domesticum	8280^T	AF075512
Trichosporon dulcitum	8257^T	AF075517
Trichosporon faecale	4828^T	AF105395
Trichosporon gracile	8189^T	AF105399
Trichosporon jirovecii	6864^T	AF105398
Trichosporon inkin	5585^T	AF105396
Trichosporon laibachii	5790^T	AF075514
Trichosporon loubieri	7065^T	AF075522
Trichosporon moniliiforme	2467^T	AF105392
Trichosporon montevideense	6721^T	AF105397
Trichosporon mucoides	7625^T	AF075515
Trichosporon multisporum	2495^T	AF139984
Trichosporon ovoides	7556^T	AF075523
Trichosporon pullulans	2532^T	AF105394
Trichosporon veenhuisii	7136^T	AF105400
Udeniomyces megalosporus	7236^T	AF075510
Udeniomyces pyricola	6754^T	AF075507

method (Middelhoven *et al*., 1991). The pH of growth media was adjusted to 5·5 if required, but the pH of media with galacturonic or quinic acid or potassium hemi-saccharate was not adjusted, which is in agreement with the laboratory practice of CBS Delft (D. Yarrow, personal communication; cf. Middelhoven, 1997). Assimilation of carbon compounds other than those used in the standard species description was studied in a synthetic growth medium (Middelhoven *et al*., 1991), which differed from Difco YNB by a tenfold higher potassium phosphate concentration and by a lower concentration of ammonium chloride, 2 rather than 6 g l^{-1}. Assimilation of potentially toxic benzene compounds listed in Table 5 was studied by the slant culture method (Middelhoven *et al*., 1991).

Sequence analysis. Strains (Table 1) were obtained from CBS, Delft, The Netherlands, and all molecular sequencing and analyses were performed at the University of Miami using the following procedures. Cells from pure cultures were grown for 12–14 h in GYP (2% glucose, 0·5% peptone and 0·1% yeast extract), then centrifuged/washed with distilled water and converted into spheroplasts by incubating for 2 h at 37 °C in 10 mM citrate buffer, pH 5·8, 1 M sorbitol and lysing enzymes (10 mg ml^{-1}) from *Trichoderma harzianum* (Sigma), which was freshly prepared for each extraction procedure. DNA was purified from the spheroplasts using the QIAamp tissue culture kit (Qiagen) following the standard protocol. The DNA was amplified with universal fungal primers ITS5 (5′-CGA AGT AAA GTC GTA ACA AAG G) and LR6 (5′-CGC CAG TTC TGC TTA CC) using MJ Research Thermal Cyclers. The resulting amplicon was purified with the QIAquick PCR purification kit (Qiagen). Cycle sequencing of the D1/D2 600–650 bp region at the 5′ end of the LSU employed forward primer F63 (5′-GCA TAT CAA TAA GCG GAG GAA AAG) and reverse primer LR3 (5′-GGT CCG TGT TTC AAG ACG). Nucleotide sequences were obtained using standard LiCor protocol with IRD800 conjugate primers and a LiCor automated sequencer. Sequences were aligned with Megalign (DNAstar) and visually corrected. Phylogenetic analysis employed PAUP* 4.0 using parsimony analysis, random step-wise addition, tree bisection–reconnection. Gaps were handled as missing data. Complete sequences are available in GenBank (Table 1).

RESULTS

Latin diagnosis of *Trichosporon veenhuisii* Middelhoven, Scorzetti *et* Fell sp. nov.

In medio liquido dextrosum et peptonum et extractum levidinis continente post 3 *dies ad* 20 °C *cellulae ovoideae* (2·6–3·8 × 4·4–16·5 μm), *singulae vel binae. Sedimentum et pellicula repens et pseudomycelium formantur. Etiam post* 4 *hebdomades sedimentum et pellicula adsunt. Cultura in agaro extracta malti et faecis continente post* 3 *dies cremea, lucida, butyrosa, rugosa, elevata et mycelio fimbriata; post hebdomades*

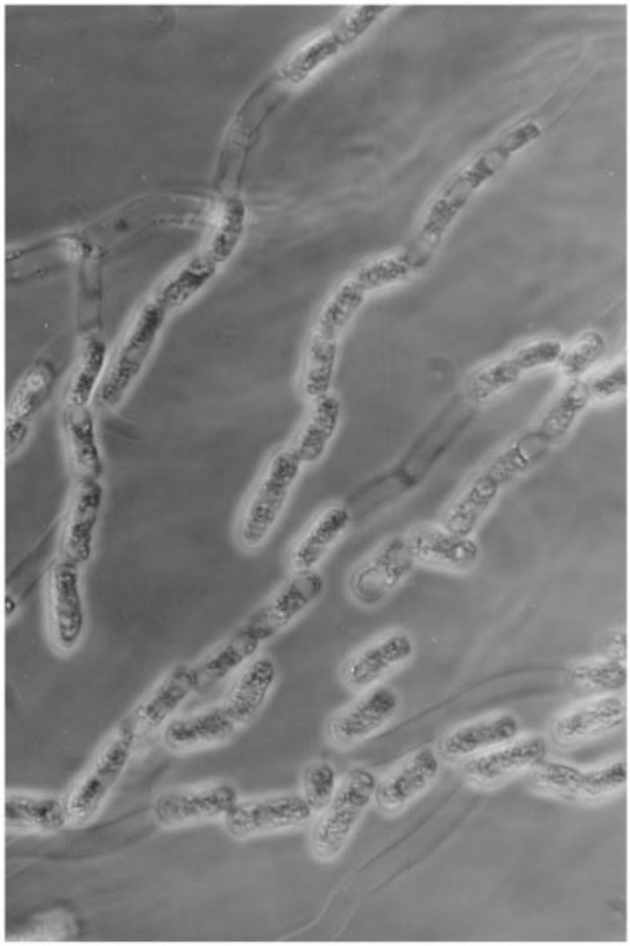

Fig. 1. *T. veenhuisii* CBS 7136^T after 3 d at 25 °C in a slide culture on Difco corn meal agar.

Table 2. Assimilation of carbon compounds by *T. veenhuisii* CBS 7136^T

D, Delayed positive (after 14 d or more); DW, delayed and weak.

Compound	Reaction	Compound	Reaction
D-Glucose	+	Ribitol	DW
D-Galactose	D	Xylitol	−
L-Sorbose	−	L-Arabinitol	D
D-Glucosamine	DW	D-Glucitol	D
D-Ribose	+	D-Mannitol	D
D-Xylose	+	Galactitol	−
L-Arabinose	+	*myo*-Inositol	+
D-Arabinose	−	D-Glucono-1,5-lactone	−
L-Rhamnose	−	2-Keto-D-gluconate	+
Sucrose	+	5-Keto-D-gluconate	+
Maltose	+	D-Gluconate	+
αα-Trehalose	+	D-Glucuronate	+
Methyl α-D-glucoside	+	D-Galacturonic acid	−
Cellobiose	+	DL-Lactate	+
Salicin	−	Succinate	+
Arbutin	+	Citrate	−
Melibiose	−	Methanol	−
Lactose	+	Ethanol	+
Raffinose	D	Propane-1,2-diol	−
Melezitose	DW	Butane-2,3-diol	+
Inulin	−	Quinic acid	−
Soluble starch	DW	Saccharate	−
Glycerol	+	Galactonate	D
Erythritol	−	Acetyl-D-glucosamine	+

4, 20 °C, *eadem forma nec lucida*. *In agaro farina Zeae maydis confecto post dies* 3, 20 °C, *mycelium et pseudomycelium formantur*. *Catenae athroconidiorum formantur*. *Fermentatio nulla*. *D-Glucosum*, *D-galactosum* (*lente*), *D-glucosaminum* (*lente*), *acetyl-D-glucosaminum*, *D-ribosum*, *D-xylosum*, *L-arabinosum*, *sucrosum*, *maltosum*, *trehalosum*, *methyl α-D-glucosidum*, *cellobiosum*, *arbutinum*, *lactosum*, *raffinosum* (*lente*), *melezitosum* (*lente*), *amylum solubile* (*lente*), *glycerolum*, *ribitolum* (*lente*), *L-arabitolum* (*lente*), *D-glucitolum* (*lente*), *D-mannitolum* (*lente*), *inositolum*, *acidum* 2-*keto-D-gluconicum*, *acidum* 5-*keto-D-gluconicum*, *acidum gluconicum*, *acidum glucuronicum*, *acidum lacticum*, *acidum succinicum*, *ethanolum*, *butano*-2,3-*diolum*, *acidum galactonicum* (*lente*) *et n-hexadecanum assimilantur*. *L-Sorbosum*, *D-arabinosum*, *L-rhamnosum*, *salicinum*, *melibiosum*, *inulinum*, *erythritolum*, *xylitolum*, *galactitolum*, *gluconolactonum*, *acidum galacturonicum*, *acidum citricum*, *methanolum*, *propano*-1,2-*diolum*, *acidum quinicum et acidum saccharicum non assimilantur*. *Ethylaminum*, *L-lysinum*, *cadaverinum et cratininum* (*lente*) *assimilantur*. *Kalii nitratum*, *sodii nitritum*, *creatinum*, *glucosaminum et imidazolum non assimilantur*. *Thiaminum externum ad crescentiam necessarium est*. 41 °C *crescit neque* 45 °C. *Ureum finditur*. *Crescere potest* 0·1 g/*litrum cycloheximidi*. *Materia amyloidea formatur* (*lente*). *Typus* CBS 7136^T *isolatus ex stercore exciccato bovis propre Minturnum*, *in collectione zymotica* Centraalbureau voor Schimmelcultures, Delft, The Netherlands.

Description of *Trichosporon veenhuisii* Middelhoven, Scorzetti *et* Fell sp. nov.

Trichosporon veenhuisii (veen.huis.i.i. M.L. n. *veenhuisii* in honour of Marten P. Veenhuis, State University of Groningen, The Netherlands, distinguished electron microscopist and author of many papers dealing with the ultrastructure of the yeast cell wall, the septal pores and, in particular, the physiological function, ultrastructure and biogenesis of peroxisomes and other microbodies occurring in the yeast cell).

Morphological characteristics of the species. After 3 d growth in glucose (2%, w/v), yeast extract (0·5%, w/v), peptone (1·0 %, w/v) broth at 25 °C the cells are ovate and long-ovate, 2·6–3·8 × 4·4–16·5 μm. Budding yeast cells and some pseudomycelium are present. A sediment and a creeping pellicle are formed. After 4 weeks a heavy sediment and a creeping pellicle are still present. The slant culture on YM agar after 3 d at 25 °C is butyrous, cream, glistening, wrinkled, raised and fringed with mycelium. After 4 weeks this culture is dull. In the slide culture on corn meal agar after 3 d at 25 °C abundant mycelium is formed, which fragments into chains of arthroconidia (Fig. 1).

Table 3. Assimilation of nitrogenous compounds by *T. veenhuisii* CBS 7136^T

Compound	Reaction
Nitrate	−
Nitrite	−
Ethylamine	+
L-Lysine	+
Cadaverine	+
Creatine	−
Creatinine	−
Glucosamine	−
Imidazole	−

Alcohol fermentation. No detectable gas is formed during fermentation of glucose.

Assimilation of carbon and nitrogen compounds. Growth responses on standard carbon and nitrogen compounds are shown in Tables 2 and 3, respectively. The assimilation of aliphatic compounds, which are not included in the usual taxonomic growth tests, is shown in Table 4; the assimilation of benzene compounds is shown in Table 5.

Other characteristics. The vitamin requirement is met by thiamin. Growth is positive at 41 °C but not at 45 °C. No growth occurred in 50% glucose/yeast extract agar. Growth in the presence of 0·1% cycloheximide was delayed, but 0·01% of this antibiotic was tolerated. No growth occurred in the presence of 10% sodium chloride. Urea was hydrolysed. The colour reaction with Diazonium Blue B was positive. Amyloid compounds appeared to be produced, but the characteristic blue colour developed some hours after addition of the iodine reagent, and was weak. Identity of the blue pigment with the product formed from amylose is doubtful.

Table 5. Assimilation of some benzene compounds as sole source of carbon and energy by *T. veenhuisii* CBS 7136^T

Slant culture method. The substrates are listed in order of the length of their side chains. D, Delayed positive (after 14 d or more); D+, positive or delayed positive, but in most cases delayed; V, variable results (positive or negative).

Compound	Reaction
Phenol	+
Hydroquinone	+
Resorcinol	+
Phloroglucinol	−
m-Cresol	+
4-Methylcatechol	+
Orcinol	−
Salicylate	−
2,3-Dihydroxybenzoate	D
2,4-Dihydroxybenzoate	−
Gallate	D+
4-Ethylphenol	−
1-Phenylethanol	+
Acetophenone	+
4-Hydroxyacetophenone	V
Tyramine	V
2-Hydroxyphenylacetate	+
3-Hydroxyphenylacetate	+
4-Hydroxyphenylacetate	+
3-Hydroxycinnamate	V
4-Hydroxycinnamate	−

Sequence analysis of the D1/D2 region of LSU. ***T. veenhuisii*** is a member of Sugita & Nakse (1998) Group III

Table 4. Assimilation of some aliphatic compounds as sole source of carbon and energy by *T. laibachii* CBS 5790^T, *T. loubieri* CBS 7065^T and CBS 8265, and *T. veenhuisii* CBS 7136^T

−,DW, Negative or delayed weak; +D, positive or delayed positive; V, variable results (positive or negative).

Compound	Reaction		
	T. laibachii	*T. veenhuisii*	*T. loubieri*
n-Hexadecane*	−,DW	+	−,DW
Uric acid	+	+	−
Glycine	+	+D	−
Ethylamine	V	−	−
n-Butylamine	+	+	+
Xylan	V	−	V
Polygalacturonate	+	−	+
Pectin	V	−	+D

* Slant culture method.

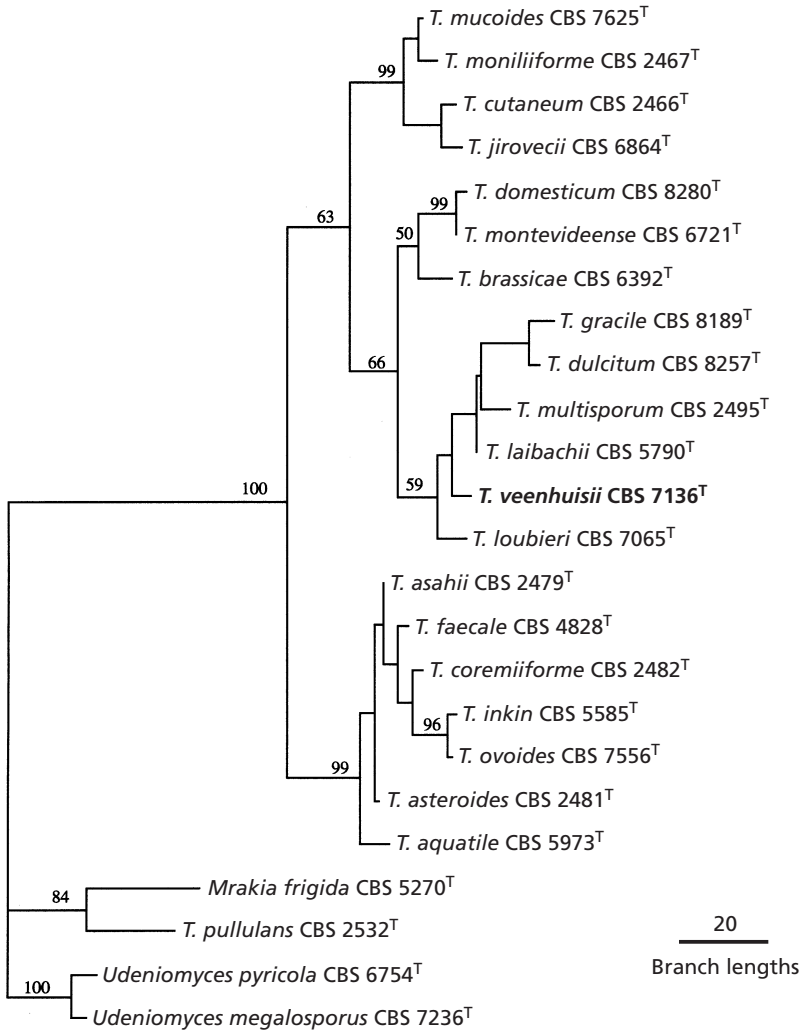

Fig. 2. Phylogenetic tree of the genus *Trichosporon* Behrend based on parsimony analysis (PAUP* 4.0; heuristic search, random step-wise addition, tree bisection–reconnection) of a ~650 bp region at the 5′ end of the LSU. Numbers at branches represent bootstrap percentages (1000 replicates). *Mrakia frigida*, *Trichosporon pullulans*, *Udenomyces pyricola* and *Udenomyces megalosporus* were used as the outgroup. T, Type strain.

species (Fig. 2). *T. veenhuisii* differs from the closest relatives *Trichosporon loubieri* (Morenz) Weijman at 11 of 625 base positions (1·8 %), *Trichosporon laibachii* (Windisch) Guého *et* M. Th. Smith at nine positions (1·4 %) and *Trichosporon multisporum* Cochet at 16 positions (2·6 %).

Origin and deposits. CBS 7136^T was isolated in August 1984 from buffalo dung collected in Minturno, Province of Campania, Italy. The type strain *T. veenhuisii* CBS 7136^T has been deposited in the yeast collection of the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, and in the culture collection of the Laboratory of Microbiology, Wageningen, The Netherlands.

DISCUSSION

The genus *Trichosporon*, as envisioned by SSU sequence analysis (Sugita & Nakase, 1998), consists of three major groups. Our results by LSU sequence analysis concur with that species group concept (Fig. 2). Differences between the Sugita & Nakase (1998) phylogenetic tree and Fig. 2 are largely due to branch arrangements. *T. veenhuisii* belongs to a cluster (bootstrap support = 59 %) of species, *Trichosporon gracile*, *Trichosporon dulcitum*, *T. multisporum*, *T. laibachii* and *T. loubieri*, which have coenzyme Q_9 as their major ubiquinone. Although coenzyme Q analysis was not available to our laboratories, *T. veenhuisii* is predicted to be positive for the presence of coenzyme Q_9. These *Trichosporon* species are soil- or animal-associated and, as far as known, are not responsible for animal or human diseases as reported for many of the other species of *Trichosporon* (Guého *et al.*, 1998; Kwon-Chung & Bennett, 1992).

Taxonomic definition of *Trichosporon* is generally based on phenotypic characters. Guého *et al.* (1992) demonstrated, based on DNA hybridization studies, that species of *Trichosporon* can be distinguished based on LSU sequence analysis. The number of base pair differences required to separate closely related species among yeasts has not been established and will possibly vary between phylogenetic groups. A view of the combined data from our study (base position differences) and from Guého *et al.* (1992) (percentage relative binding of DNA) does provide some evidence.

Table 6. Characteristics that differentiate *T. laibachii*, *T. veenhuisii* and *T. loubieri*

D, Delayed positive (after 14 d or more); DW, delayed and weak; −,D, negative sometimes delayed positive; +,D, positive or delayed positive; V, variable results (positive or negative).

	*T. laibachii**	*T. veenhuisii*	*T. loubieri**
L-Sorbose	+	−	V
D-Glucosamine	+	DW	+
L-Rhamnose	+	−	+
Salicin	+	−	+
Melibiose	+	−	+
Soluble starch	+	DW	+
Ribitol	−	DW	+
L-Arabinitol	−,D	D	+
Galactitol	+	−	−
Gluconolactone	V	−	+
Citrate	+	−	+
Propane-1,2-diol	+	−	+
Butane-2,3-diol	−,D	+	+,D
Quinic acid	+	−	+
Galactonate	+	D	+
Creatinine	+	−	−
Max. growth temp. (°C)	<35	41	42/45

* Data from the CBS Yeasts Database (http://www.cbs.knaw.nl).

As a point of reference, Guého *et al*. (1992) consider reassociation values above 80% to be proof of conspecificity. For example, DNA hybridization between multiple strains of *Trichosporon mucoides* Guého *et* M. Th. Smith and *Trichosporon moniliiforme* (Aciole de Queiros) Guého *et* M. Th. Smith is 0–29% and base differences between the type strains of the two species is 4/625 base positions (0·5%). In other examples, *Trichosporon inkin* (Oho *ex* Ota) do Carmo-Sousa *et* van Uden and *Trichosporon ovoides* Behrend DNA hybridization is 12–53% and base pair position differences are 0·3 %. *T. loubieri* and *T. laibachii* differ at 2·2% base positions with a DNA hybridization value of 53%. Although DNA hybridization tests have not been carried out with *T. veenhuisii*, due to a lack of availability of the procedure in our laboratories, the base position differences are within the range for species separations. Specifically, *T. veenhuisii* differs from *T. loubieri* by 1·8%, *T. laibachii* by 1·4% and *T. multisporum* by 2·6%.

T. veenhuisii CBS 7136^T was phenotypically notable within the genus for specific growth characteristics and utilization of carbon sources. In particular, *T. veenhuisii* demonstrated dense growth in slant cultures with n-hexadecane, which usually became visible within 2 weeks at 25 °C. Of all other 21 species tested only *T. aquatile* (Barnett *et al*., 1983) and some strains of *Trichosporon asahii* Akagi *ex* Sugita, Nishikawa *et* Shinoda (unpublished results) showed this character. Strains of some other species, *T. laibachii* and *T. loubieri* included, showed variable, weak and slow growth on this hydrocarbon (unpublished results). Some *Trichosporon* species assimilated many benzene compounds (Middelhoven, 1993). *T. veenhuisii* CBS 7136^T also showed this degradative ability (Table 5). In contrast to *T. laibachii* and *T. loubieri*, *T. veenhuisii* CBS 7136^T was unable to assimilate polysaccharides like xylan, polygalacturonate and pectin (Table 4).

Like most other *Trichosporon* species, *T. veenhuisii* CBS 7136^T assimilated many of the carbon sources used in the taxonomic growth tests (Table 1). *T. veenhuisii* can be distinguished from *T. laibachii* and *T. loubieri* by the failure to assimilate L-sorbose, L-rhamnose, salicin, melibiose, citric and quinic acids and propane-1,2-diol (Table 6).

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