

The cell wall of the oleaginous yeast *Trichosporon cutaneum*

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The cell wall of *Trichosporon cutaneum* consists of 11 % protein, 63 % neutral carbohydrate, 9 % glucosamine and 13 % glucuronic acid. The sugars include glucose (32 %), mannose (6 %) and traces of xylose and galactose. The cell wall was fractionated with alkali to yield a mixture of alkali-soluble matrix components, and an alkali-insoluble glucan associated with chitin. The alkali-insoluble glucan contained a mixture of (1-3) and (1-6) glycosidic linkages. It was only partly susceptible to digestion by the β (1-3) glucanase, Zymolyase. The alkali-soluble fraction contained glucan, mannan and acidic polymers. The glucan was (1-3)-linked with no (1-6) linkages and only trace amounts of (1-3-6)-linked glucose. It was resistant to digestion by Zymolyase. Extensive hydrolysis of this fraction with trifluoroacetic acid released a high-molecular-mass glucuronan which had ^1H - and ^{13}C -NMR profiles matching those of the β (1-4) glucuronan, mucoric acid. Xylomannan was purified from isolated cell walls and from whole cells. It contained glucose, mannose, xylose, and D-glucuronic acid. It was very similar in composition and structure to the capsular polysaccharides of *Cryptococcus neoformans*, and to an extracellular polysaccharide produced by another yeast described as *T. cutaneum*. Electron microscopy showed that the cell wall of *T. cutaneum* has a lamellar structure characteristic of a basidiomycetous yeast rather than the electron-dense 'fuzzy coat' seen in *Candida albicans*.

Introduction

The organism here referred to as *Trichosporon cutaneum* was first isolated from the floor drain of a dairy factory and classified as *Candida curvata* D (Moon *et al.*, 1978). It converts carbohydrate to edible oil with high efficiency (Boulton & Ratledge, 1981) and unlike most yeasts (Barnett, 1981), it utilizes lactose as a carbon source. The initial step of lactose utilization by *T. cutaneum* has been studied by West *et al.* (1990).

von Arx & Weijman (1979) reclassified the basidiomycetous species of *Candida* as *Apiotrichum* or *Rhodotorula*, based on the presence or absence, respectively, of xylose in the cell wall. In this way the organism that had been identified as *Candida curvata* D was reclassified as *Apiotrichum curvatum* ATCC 20509. Subsequently it was examined at The National Collection Of Yeast Cultures, Norwich UK (J. Davies, personal communication). Well-developed pseudomycelium, limited true mycelium and arthrospores were produced in plate cultures and the biochemical features of the isolate agreed with the standard description of *T. cutaneum* (Kreger-van Rij, 1984). True mycelium and arthrospores are characteristic

of the genus *Trichosporon* but not of the genus *Candida*, which means that the initial classification of the organism as *Candida curvata* D and its reclassification as *Apiotrichum* were invalid. The reclassification of this organism as *T. cutaneum* presents a problem however, in that a diverse mix of organisms have been given this name. One strain of *T. cutaneum*, isolated from raw sewage, produces an extracellular pentosyl mannan (Gorin & Spencer, 1967), another strain isolated from soil is able to use phenol as a substrate (Spånnig & Neujahr, 1990). Some papers refer to an organism described as *Trichosporon beigeli* [= *T. cutaneum* (Guého *et al.*, 1987; Kreger-van Rij, 1984)] which has been shown to cause systemic infections in humans (Matthews *et al.*, 1986). Bearing all this in mind, the organism *Apiotrichum curvatum* ATCC 20509 will be referred to in this study as *T. cutaneum*.

Although the biochemistry of oil production and the mechanism by which this organism utilizes lactose have been studied in detail, little is known of the basic biology of *T. cutaneum*. This study of the cell wall composition and structure was undertaken both to extend the understanding of the biology and classification of this organism, and to provide basic information to aid in the design of protocols for oil extraction on an industrial scale.

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Methods

Cultures and growth conditions. *Trichosporon cutaneum* (*Apiotrichum curvatum* ATCC 20509) was obtained from Industrial Processing Division, DSIR, Wellington, New Zealand, and maintained as slope cultures on malt extract agar (Difco) at 4 °C.

Cultures were grown on YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) at 28 °C as 10 litre cultures in a New Brunswick model SF-116 fermenter; agitation 400 r.p.m., aeration 10 l min⁻¹, with 1–2 ml antifoam compound A (Sigma). These cultures were inoculated with 500 ml of a YPD culture which had been grown on a shaker for 16 h at 28 °C.

Analytical methods. Total neutral carbohydrate was measured using the phenol sulphuric assay (Dubois *et al.*, 1956), with a glucose standard unless otherwise noted. The colour response per mg glucuronic acid and mannose at 488 nm was 42% and 142%, respectively, of the response of glucose. All assays of neutral carbohydrate have been corrected for the presence of uronic acid where appropriate. Protein was assayed by a modified Lowry assay (Miller & Hoskins, 1981), using the reagents described by Eggstein & Kreutz (1967) and BSA as a standard. Amino sugar was released by hydrolysis for 18 h in 6 M-HCl at 110 °C under vacuum, and measured by a modification of the Morgan-Elson assay (Ghosh & Roseman, 1962). Using this method, it was possible to account for 85% of the dry wt of crab-shell chitin (purified according to Jeuniaux, 1966) as glucosamine. Reducing sugar was measured by reaction with *p*-hydroxybenzoic acid hydrazide (Lever, 1973). Uronic acid was measured using the carbazole reaction (Bitter & Muir, 1962) with a standard of glucuronic acid. To correct for interference by neutral sugars, control samples were treated as specified by Bitter & Muir (1962) except that they were heated with ethanol instead of carbazole in ethanol. Uronic acids were converted to the corresponding neutral sugars by reduction with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide HCl and sodium borohydride as specified by Redgewell *et al.*, (1988) except that denaturation of the polysaccharide with urea was not required. Glucose was measured by the glucose oxidase assay (Lloyd & Whelan, 1969). The cell dry wt was measured by pipetting 1 ml samples of 1:1 wet wt/vol. cell suspension into tared 5 ml borosilicate tubes, drying overnight at 100 °C and allowing them to cool in a desiccator.

All assays were performed in triplicate unless otherwise noted.

Preparation of cell walls. YPD cultures (10 l) grown for 70 h yielded 800 g wet wt cells (dry wt 110 g). The washed cells, suspended in water (1:1 wet wt/vol.) were broken in a Manton Gaulin homogenizer [6–7 passes at 12000 p.s.i. (83 MPa)] or in a French Press (Aminco Maryland; two to three passes at 20000 p.s.i.). Breakage assessed by light microscopy was > 95% for the Manton Gaulin and > 98% for the French Press. The cell-wall fraction recovered by centrifuging at 16000 g was washed successively in 0.9% NaCl (four times), water (four times), ethanol (twice), chloroform/methanol (1:1, v/v) (twice), ethanol (twice), and again in 0.9% NaCl and water as above. The final yield, 12 g dry wt, represented 10–12% of the cell dry wt.

Alkali extraction of dry cell walls. Samples of dry cell wall (1 g) were extracted in 250 ml 3% (w/v) NaOH at 4 °C for 6 d with stirring (Fleet, 1985). The insoluble fraction was recovered by centrifuging (27000 g for 30 min) and was washed once with fresh alkali.

The combined supernatant and washings were adjusted to pH 6.4 with 6 M-acetic acid and allowed to stand at 4 °C but no precipitate formed after 16 h. Ethanol (4 vols) was then added, the precipitate was collected, washed with ethanol and dried *in vacuo* over P₂O₅.

The pellet from the original alkali extraction was resuspended in 100 ml water, adjusted to pH 6.0 with acetic acid and centrifuged. No carbohydrate was detected in the supernatant by the phenol sulphuric assay. The pellet was washed four to five times with ethanol and dried *in vacuo* over P₂O₅ as above.

Gas-liquid chromatography. Samples (5–10 mg) were hydrolysed at 100 °C for 2 h in 2 M-trifluoroacetic acid with erythritol (0.5–1 mg) as an internal standard. Trifluoroacetic acid was removed by drying the samples repeatedly *in vacuo*. Alditol acetates were prepared according to Blakeney *et al.* (1983) and stored in 1 ml dichloromethane. Typically 1–5 µl samples were analysed in a Hewlett Packard 5890A gas chromatograph fitted with a BP-X70 column and flame ionization detector (Sawardeker *et al.*, 1965). The column head pressure was 3 p.s.i., flow of hydrogen was 1 ml per 3 s. The column was held at an initial temperature of 180 °C for 5 min, then the temperature was increased at a rate of 3 °C min⁻¹ to a final temperature of 230 °C and maintained for 10 min. The injection temperature was 240 °C, the detector temperature was 280 °C. Sugars were identified by comparison of the retention times with those of standards and relative amounts were calculated using a Shimadzu C-R3A Chromatopac recorder with integrator. The limit of detection was approximately 0.2 µg.

Methylation. Sodium dimethyl was prepared according to the method of Sanford & Conrad (1966). Samples (20 mg) were weighed into dry scintillation vials capped with serum stoppers, flushed with dry N₂ for 1 h and dissolved in dry DMSO (4 ml) by heating at 40–70 °C in an oil bath with stirring. The samples were reacted overnight with 1.5 ml sodium dimethyl and methylated with 1.5 ml methyl iodide as specified by Stoffel & Hanflan (1973). They were then freeze-dried, extracted into chloroform and dried *in vacuo*. Samples were checked for active dimethyl by reaction with triphenylmethane before addition of methyl iodide (Rauvala, 1979). Each sample was methylated three times. The alkali-insoluble fraction was dispersed in DMSO by sonication (5–6 30 min cycles) and approximately 20% of the starting material became soluble in DMSO after one cycle of methylation. The remainder was discarded.

Each methylated polysaccharide was dissolved in 0.125 ml 13 M-H₂SO₄ at room temperature for 45 min, 1.35 ml of water was added and samples were hydrolysed at 110 °C for 5 h. Hydrolysates were neutralized with solid BaCO₃, and the insoluble BaSO₄ removed by centrifugation. Methylated alditol acetates were prepared as follows (J. Tamate, personal communication): the solution containing methyl sugars was concentrated to a minimal volume (approx. 0.1 ml) on a rotary evaporator (water bath temperature ≤ 30 °C), made up to a total of 1 ml with 2% (w/v) sodium borohydride and allowed to stand overnight. The excess borohydride was decomposed with glacial acetic acid and the solution was concentrated to a minimal volume as above. The residue was taken up in 2 ml methanol and dried under nitrogen four times. The methylated sugars were dissolved in 1 ml dichloromethane and the amorphous precipitate of sodium acetate was discarded. 1-Methyl-imidazole (0.1 ml) and acetic anhydride (0.2 ml) were added and samples were heated at 50–60 °C in a water bath for 30 min. Finally the methylated derivatives in dichloromethane were washed twice with 2 ml water, dried with anhydrous Na₂SO₄ and stored at –20 °C in Teflon-capped vials.

Methylated alditol acetates were separated using a Hewlett Packard 5890A gas chromatograph fitted with a BP-1 column. Conditions were: column head pressure 10 p.s.i. (69 kPa), flow of hydrogen 1 ml per 3 s. The temperature was held at 100 °C, for 5 min after injection of sample, then increased by 4 °C min⁻¹ to 230 °C which was maintained for 10 min. The injection temperature was 230 °C, detector temperature 280 °C. Samples for mass spectroscopy were assayed on a Perkin Elmer 8420 GC fitted with a 12 m DB-1 column and connected to a Perkin Elmer ITD Bench top Mass Spectrometer; conditions as for the BP-1 column. Peaks were identified by comparison of fragmentation patterns with those reported by Jansson *et al.* (1976).

Digestion of fractions with enzymes. Isolated walls or wall fractions (10 mg) were suspended in 2 ml 10 mM-sodium citrate buffer (pH as below) and autoclaved at 120 °C for 20 min to prevent bacterial growth. The samples were then incubated at 30 °C with 1 mg Novozyme 234 (pH 5.8) or Zymolyase 20T (pH 6.5) for 48 h. Control samples of

wall or fractions thereof were incubated in buffer containing no enzyme. Reducing sugar was assayed by the method of Lever (1973) and *N*-acetyl glucosamine by the modified Morgan-Elson reaction as described previously. No additional release of either reducing sugar or *N*-acetyl glucosamine was observed when the residues were incubated with 10 mg Novozyme 234 or 2 mg Zymolyase 100T. Novozyme 234 was obtained from Novo Biolabs, Zymolyase 20T and 100T were obtained from Seikagaku Kogyo.

Chromatography of mannoproteins. Samples were dissolved in 10–50 ml 10 mM-potassium phosphate buffer, pH 6.0 and loaded onto a 32 × 5 cm column of DEAE Sephadex equilibrated with the same buffer. Unbound material was eluted with 1.6 l potassium phosphate buffer, pH 6.0 at a flow rate of 2 ml min⁻¹. Mannoproteins were eluted with a 2 litre linear gradient of 0–1 M-NaCl in 10 mM-potassium phosphate buffer, pH 6.0. Fractions (10 ml) were collected and assayed for carbohydrate by the phenol sulphuric assay and for protein by measurement of A_{280} .

Samples up to 40 mg were dissolved in 2 ml 0.5 M-NaCl and loaded onto a 102 × 1.5 cm column of Sephacryl S-300 with 2 cm × 1.5 cm plugs of Biogel P2 at each end. Fractions (2 ml) were collected and assayed for protein by measurement of A_{280} and for carbohydrate by the phenol sulphuric assay.

Extraction and partial purification of mannoprotein from whole cells. Washed cells from a 10 l culture of *T. cutaneum*, grown for 40 h were suspended in 10 mM-sodium citrate buffer pH 7.0 [1 ml (g wet wt)⁻¹], and autoclaved for 40 min at 120 °C. The insoluble residue was collected by centrifugation, re-extracted with fresh sodium citrate buffer and centrifuged again. The combined citrate extracts were added to 5 vols 95% (v/v) ethanol and stored at 4 °C overnight. The precipitate was redissolved in 500 ml water and dialysed for 2 h against 5 l water at 4 °C. Ammonium sulphate was added to 50% saturation and the solution was allowed to stand for 1.5 h at 4 °C. The precipitate was collected by centrifugation, resuspended in 300 ml water for 1 h and centrifuged again. The supernatant was dialysed extensively against tap water to remove the ammonium sulphate and freeze-dried. The freeze-dried material (5.31 g) was dissolved in 100 ml water, clarified by centrifugation and freeze-dried again.

NMR. ¹³C-NMR spectra were obtained with a Varian Gemini-200 NMR Spectrometer calibrated with an external standard of tertiary butanol in a coaxial capillary. Samples were dissolved in 0.6 ml D₂O. The temperature was 25 °C, the frequency was 50 MHz and a total of 100 000 transients were used. In each run the acquisition time was 1.0 s, the delay was 1.0 s and the pulse width was 29 ms. The assignment of peaks was made by comparison with spectra reported for mucoric acid (Tsuchihashi *et al.*, 1983).

¹H-NMR spectra were obtained from the same samples used for ¹³C-NMR spectroscopy using a Varian VXR-300 NMR spectrometer at a temperature of 75 °C with methanol as an internal standard. The frequency was 300 MHz and a total of 80 transients were required. In each run the acquisition time was 1.0 s, the delay was 1.0 s and the pulse width was 24.2 ms.

Results

Fractionation of the wall with alkali

The wall preparation was fractionated with alkali to yield soluble and insoluble fractions equal to 34% and 44%, respectively, (i.e. 78% of the dry wt) of the original cell walls. Possible reasons for this low recovery include limited degradation of the cell wall constituents, despite the mild extraction conditions, and losses during the

ethanol precipitation of the alkali-soluble fraction. Yields of 83–84% (based on estimates of carbohydrate, protein and lipid) have been obtained from a similar fractionation of the walls of *Schizosaccharomyces pombe* (Manners & Meyer, 1977) and 84–94% from cells of *Candida albicans* (Sullivan *et al.*, 1983).

Chemical analysis of the whole wall and fractions

The major constituent of the wall was neutral carbohydrate (63%) (Table 1). Other components were: protein (11%), glucosamine (9%) and uronic acid (13%). The content of neutral carbohydrate is much less than is found in the walls of *Saccharomyces cerevisiae* (80–90%) (Fleet, 1985) or *C. albicans* (80%) (Sullivan *et al.*, 1983), but is comparable to that in the walls of filamentous fungi, such as *Schizophyllum commune* (71%) (Sietsma & Wessels, 1977) and *Trichoderma viride* (30–40%) (Benitez *et al.*, 1975). The amount of chitin (based on glucosamine) in the wall of *T. cutaneum* is also comparable to that found in *Schizophyllum commune* (13%). The composition of the wall of *T. cutaneum* is therefore more consistent with that of a filamentous fungus than a yeast.

Hydrolysates of whole cells of *T. beigelii* contain uronic acid (Weijman, 1979) but, as far as we are aware, this is the first time that it has been described as a genuine wall component of a species of *Trichosporon*. The amount of glucuronic acid found in the cell wall of *T. cutaneum* is comparable to that in yeast cells of the Zygomycete *Mucor rouxii* (Bartnicki-Garcia & Reyes, 1968).

Samples of the wall and of fractions thereof were hydrolysed and neutral sugars were assayed by GLC (Table 2). The original wall preparation contained 32% glucose, 5–6% mannose and traces of xylose and galactose. The content of neutral sugars is likely to have been underestimated due to incomplete hydrolysis of polymers containing either glucosamine or uronic acid. A similar pattern of monosaccharides has been found in cell hydrolysates of *T. beigelii* (Weijman, 1979). The yields for the individual sugars detected by GLC, together with the data for protein, glucosamine and glucuronic acid, account for 91% of the cell wall preparation.

Analysis of the alkali-insoluble fraction

The alkali-insoluble fraction contained 56% neutral carbohydrate, 23% glucosamine, 7% glucuronic acid and a trace of protein (Table 1). Analysis of the neutral sugars after hydrolysis (Table 2) revealed glucose as the main component together with traces of mannose and galactose. Digestion of this fraction with Zymolyase (a lytic enzyme preparation containing a mixture of $\beta(1-3)$

Table 1. *Percentage composition of the wall and fractions*

The cell wall preparation was fractionated into alkali-soluble and -insoluble material which comprised 34 and 44%, respectively, of the wall on a per weight basis. Protein, carbohydrate, glucosamine and glucuronic acid were assayed as described in Methods. Results are expressed as percentages of the total dry wt of each fraction. Figures in parentheses show the number of samples taken for each estimation. Figures for carbohydrate have been corrected for the response of glucuronic acid in the phenol sulphuric assay. Both neutral sugar and protein were destroyed under the conditions required to liberate glucosamine from chitin and did not interfere with assays of glucosamine.

	Protein	Neutral carbohydrate	Glucosamine	Glucuronic acid	Total
Whole wall	11 ± 1 (3)	63 ± 1 (3)	9 ± 2 (6)	13 ± 0.4 (5)	96
Alkali-soluble	18 ± 0.5 (3)	80 ± 1 (3)	5 ± 0.2 (6)	17 ± 1 (6)	120
Alkali-insoluble	4 ± 0.4 (3)	56 ± 4 (3)	23 ± 1 (6)	7 ± 1 (6)	90

Table 2. *Percentage of monosaccharides in hydrolysates of the wall and fractions*

Samples of each fraction (5–10 mg) were hydrolysed in acid with an internal standard of 0.5–1 mg erythritol before being converted to alditol acetates and analysed as described in Methods. All fractions contained traces of ribose and arabinose (< 0.1%). Values are given as percentages of the dry wt of each fraction.

Fraction	Glucose	Mannose	Xylose	Galactose	Total
Whole wall	32 ± 4	5.6 ± 0.9	0.9	2	41
Alkali-soluble	26 ± 4	12 ± 2	0.8	1.8 ± 0.7	40
Alkali-insoluble	55 ± 11	1.8 ± 0.7	–	3	60

glucanases; Kitamura & Yamamoto, 1972) released reducing sugar equal to 12% of the total dry wt, indicating that the alkali-insoluble fraction contains $\beta(1-3)$ glucan. The remainder was resistant to hydrolysis by Zymolyase, probably due to the disposition of (1-6) branch points along the $\beta(1-3)$ -linked glucan chains (see below). Novozyme 234 (a complex mixture of enzymes derived from *Trichoderma harzianum*, and containing glucanases and chitinase activities; Perberdy, 1979) released reducing sugar equal to at least 60% of the total dry wt.

The cell wall of a yeast or filamentous fungus may contain either chitin (a polymer of *N*-acetyl glucosamine) or chitosan (a positively charged polymer of glucosamine). In view of the high content of uronic acid in the wall, chitosan might have been expected as is seen in *Mucor* spp. (Datema *et al.*, 1977). Exhaustive digestion of both the wall and the alkali-insoluble fraction with Novozyme 234 released an amount of *N*-acetyl glucosamine equal to that released after hydrolysis with 6 M-HCl (Depree, 1992). Acetylation of these enzyme lysates (Ghosh & Roseman, 1962) did not affect the yield of *N*-acetyl glucosamine, indicating that it was derived from chitin rather than chitosan.

The alkali-insoluble fraction was sparingly soluble in

Table 3. *Methylation analysis of the alkali-insoluble fraction*

A sample of the alkali-insoluble wall was suspended in DMSO with sonication, and methylated according to Stoffel & Hanflan (1973). After one cycle of methylation approximately 20% of the starting material became soluble in DMSO. The DMSO-soluble extract was methylated three times and analysed by GLC and GLC-MS as described in Methods.

O-Methyl alditol acetate derivative	Type of linkage	Percentage composition	Molar ratio
2,3,4,6-Tetra- <i>O</i> -methyl hexitol	Terminal hexose	10.0	1.5
2,4,6-Tri- <i>O</i> -methyl hexitol	(1-3) Glucose	37.6	5.8
2,4,6-Tri- <i>O</i> -methyl hexitol	(1-3) Mannose	6.6	1.0
2,3,4-Tri- <i>O</i> -methyl hexitol	(1-6) Hexose	35.3	5.5
2,4-Di- <i>O</i> -methyl hexitol	(1-3-6) Hexose	9.6	1.4

DMSO, but approximately 20% was rendered soluble after one cycle of methylation. This fraction yielded equivalent amounts of (1-3)- and (1-6)-linked glucose residues, with a molar ratio of 1.5 terminal residues to 1.4 (1-3-6)-linked branch points (Table 3). The glucan could be either a single $\beta(1-3)$ $\beta(1-6)$ -linked polymer of the type found in *C. albicans* (Gopal *et al.*, 1984) and *Schizopyllum commune* (Sietsma & Wessels, 1977), or a mixture of a predominantly $\beta(1-3)$ -linked alkali-insoluble, acid-insoluble glucan and a predominantly $\beta(1-6)$ -linked acid-soluble glucan as seen in *Saccharomyces cerevisiae* (Fleet, 1985). The alkali-insoluble fractions contain the main structural polymers of the wall of *Saccharomyces cerevisiae* (Fleet, 1985) and possibly *Schizopyllum commune* (Sietsma *et al.*, 1985).

Analysis of the alkali-soluble fraction

The alkali-soluble fraction contained 18% protein, 80% neutral carbohydrate, 17% glucuronic acid and a small amount (5%) of glucosamine (Table 1). The figure of

Table 4. *Methylation analysis of the alkali-soluble fraction*

A sample of the alkali-soluble wall was dissolved in dry DMSO, methylated four times (Stoffel & Hanflan, 1973), hydrolysed and analysed using GLC and GLC MS as described in Methods. Peaks were assigned according to retention time and by comparison of the fragmentation patterns with published data (Jansson *et al.*, 1976).

O-Methyl alditol acetate derivative	Type of linkage	Percentage composition	Molar ratio
2,3,4-Tri-O-methyl pentitol	Terminal xylose	2.4	5
2,3-Di-O-methyl pentitol	(1-4) Xylose	0.5	1
2,3,4,6-Tetra-O-methyl hexitol	Terminal hexose	2.7	5
2,4,6-Tri-O-methyl hexitol	(1-3) Glucose	65.6	112
2,4,6-Tri-O-methyl hexitol	(1-3) Mannose	18.4	31
2,4,6-Tri-O-methyl hexitol	(1-3) Galactose	5.3	9
2,6-Di-O-methyl hexitol	(1-3-4) Hexose	2.7	4
4,6-Di-O-methyl hexitol	(1-2-3) Hexose	1.3	2
2,4-Di-O-methyl hexitol	(1-3-6) Hexose	1.1	2

Table 5. *Sugar composition of the acidic mannan fractions*

Molar proportions of glucose, mannose, xylose and glucuronic acid in mannan fractions. Neutral sugars were measured by gas chromatography, glucuronic acid was measured by the carbazole assay as detailed in Methods. The proportions of mannose in each sample have been normalized to 10. ND, Not determined.

Fraction	Glucose	Mannose	Xylose	Glucuronic acid
Mannan I	2.3	10	1.2	ND
Mannan II	1.2	10	0.5	ND
Mannan III	0.9	10	2.5	3

80% neutral carbohydrate is probably an overestimate due to the high colour yield of mannose in the phenol sulphuric assay (Dubois *et al.*, 1956). Subsequent analysis by GLC (Table 2) showed that the neutral sugar content of the alkali-soluble fraction was 40%, including glucose (26%) and mannose (12%). These are likely to have been underestimated because the alkali-soluble fraction contains uronic acid. Because the bond between a neutral sugar and uronic acid is resistant to acid hydrolysis, polymers containing both neutral and acidic sugars tend to hydrolyse incompletely to produce acidic oligosaccharides, which are not detected by GLC. Conversely xylose residues are easily destroyed during acid hydrolysis (Turner & Cherniak, 1991). If each uronic acid residue were attached to a neutral sugar, then the true content of neutral carbohydrate could be as high as 57%.

Methylation analysis (Table 4) showed that the alkali-soluble fraction contained 66% (1-3)-linked glucose, 18% (1-3)-linked mannose and 5% (1-3)-linked galactose. This fraction also contained terminal xylose (2.4%), (1-4)-linked xylose (0.5%), terminal hexose (2.7%), (1-3-4)-linked hexose (2.7%), (1-2-3)-linked hexose (1.3%) and (1-3-6)-linked hexose (1.1%). The molar ratio of terminal residues to branch residues was 5:4. Despite the large proportion of (1-3)-linked glucose in this fraction it was completely resistant to Zymolyase, either because the glucan is α -linked or because the β -linkages are protected from β -glucanases in some way. Reducing sugar equal to 18–19% of the total dry wt was released by Novozyme 234 which contains a mixture of both α - and β -glucanases.

Isolation of an acidic mannan fraction

When the alkali-soluble fraction (100 mg) was heated in 10 mM-potassium phosphate buffer, pH 6.0 a small amount of material was solubilized. This material bound to DEAE Sephadex and was eluted with a linear gradient of 0–1 M-NaCl in 10 mM-potassium phosphate buffer. Two fractions, mannan fraction I and mannan fraction II, eluted at 0.4 and 0.6 M, respectively. Both of these fractions were excluded from Sephacryl S-300 which indicates that they have M_r values of 400 000 or greater. They contained glucose, mannose, and xylose in the proportions 2.3:10:1.2, and 1.2:10:0.5 for mannan fractions I and II, respectively (Table 5). Galactose was not detected in either fraction. The yields of these fractions (3–6 mg) precluded further analysis for linkage and uronic acid content.

Mannans can be extracted by autoclaving whole cells in neutral buffer (Peat *et al.*, 1961; Nakajima & Ballou, 1974). This procedure produces large yields of mannan and avoids exposure to strong alkali (Okubo *et al.*, 1981; Fleet, 1985). Whole cells of *T. cutaneum* were autoclaved in neutral citrate buffer and the mannan was precipitated as described in Methods. This fraction was applied to DEAE Sephadex and eluted with a linear gradient of 0–1 M-NaCl. Elution was monitored with the phenol sulphuric assay. A broad peak was eluted at approximately 0.5 M-NaCl and a shoulder at approximately 0.4 M-NaCl. The eluted material was collected as one pool (mannan fraction III) and the yield, 1.7 g, represents 1.2% of the cell dry wt. This is comparable to the yield of mannans extracted with alkali from various *Trichosporon* spp. and precipitated with Fehlings solution (Gorin & Spencer, 1968). The mannan was excluded from Sephacryl S-300 which indicates that it has an M_r of 400 000 or greater; it contained only minor amounts of protein (7.5%). The molar proportions of glucose:mannose:xylose:uronic acid were: 1:10:2.5:3, similar

Table 6. Methylation analysis of *T. cutaneum* mannan

A sample of the mannan was dissolved in dry DMSO, methylated four times, hydrolysed and analysed using GLC and GLC-MS as described in Methods. Peaks were assigned according to retention time and by comparison of the fragmentation patterns with published data (Jansson *et al.*, 1976).

Derivative	Linkage	Molar Percentage
<i>(a) Unreduced polysaccharide</i>		
2,3,4-Tri- <i>O</i> -methyl pentitol	Terminal pentose	2.5
2,3-Di- <i>O</i> -methyl pentitol	(1-4) Pentose	0.8
2,3,4,6-Tetra- <i>O</i> -methyl hexitol	Terminal hexose	1.1
3,4,6-Tri- <i>O</i> -methyl hexitol	(1-2) Hexose	0.8
2,4,6-Tri- <i>O</i> -methyl hexitol	(1-3) Hexose	82
2,6-Di- <i>O</i> -methyl hexitol	(1-3-4) Hexose	4.9
4,6-Di- <i>O</i> -methyl hexitol	(1-2-3) Hexose	3.9
6-Methyl hexitol	(1-2-3-4) Hexose	0.8
2,4-Di- <i>O</i> -methyl hexitol	(1-3-6) Hexose	1.0
Hexa-acetyl hexitol	Unmethylated	1.8
<i>(b) Carboxyl-reduced polysaccharide</i>		
2,3,4-Tri- <i>O</i> -methyl pentitol	Terminal pentose	2.9
2,3-Di- <i>O</i> -methyl pentitol	(1-4) Pentose	1.6
2,3,4,6-Tetra- <i>O</i> -methyl hexitol	Terminal hexose	14.5
2,4,6-Tri- <i>O</i> -methyl hexitol	(1-3) Glucose	2.6
2,4,6-Tri- <i>O</i> -methyl hexitol	(1-3) Mannose	64.3
2,6-Di- <i>O</i> -methyl hexitol	(1-3-4) Hexose	3.4
4,6-Di- <i>O</i> -methyl hexitol	(1-2-3) Hexose	11.7

to the two mannan fractions isolated from the alkali-soluble portion of the wall (Table 5). Galactose was not detected. Differences between fractions I, II and III may be attributed to the different methods of extraction. The proportions of xylose in these fractions have probably been underestimated due to breakdown during acid hydrolysis (Turner & Cherniak, 1991). The uronic acid component was identified by reducing it to the corresponding neutral sugar as described in Methods. The reduced polysaccharide was then hydrolysed and analysed for glucose with glucose oxidase reagent. Both glucose and uronic acid were assayed before and after carboxyl reduction, and the increase in the amount of glucose detected after carboxyl reduction accounted for 70–85% of the uronic acid that had been consumed. Since the glucose oxidase assay is specific for D-glucose, these data show that the uronic acid in the mannan is D-glucuronic acid.

The pathogenic yeast *Cryptococcus neoformans* produces a mannan (Vartivarian *et al.*, 1989) and also large amounts of a capsular polysaccharide similar in composition to the acidic mannan (Cherniak *et al.*, 1991). These polymers do not however appear to be structural components of the wall as the only neutral sugar detected in isolated wall preparations was glucose (Vartivarian *et al.*, 1989). Production of capsular material or extra-cellular polysaccharides by *T. cutaneum* has been

reported (Gorin & Spencer, 1967), but was not observed with this strain. The isolation of similar mannan fractions from both isolated cell walls and whole cells suggests that this acidic mannan is a true component of the cell wall.

Linkage analysis of the *T. cutaneum* acidic mannan

The structural mannoproteins of *Saccharomyces cerevisiae* and *C. albicans* (Ballou, 1982; Saxena *et al.*, 1989) have an $\alpha(1-6)$ mannose backbone. The $\alpha(1-6)$ bonds can be specifically cleaved by acetolysis and the products separated by gel filtration to yield 'fingerprints' of manno-oligosaccharides which are specific to different species of yeast (Kocourek & Ballou, 1969). The acidic mannan of *T. cutaneum* however was resistant to acetolysis which indicates that it lacks an $\alpha(1-6)$ mannose backbone.

Samples of mannan were methylated three times and analysed by gas chromatography and mass spectroscopy (Table 6a). The hydrolysates contained predominantly (82%) 2,4,6-tri-*O*-methyl hexitol [(1-3)-linked], with no 2,3,5-tri-*O*-methyl hexitol [(1-6)-linked] and only traces of 2,4-di-*O*-methyl hexitol [(1-3-6) branch points] as expected. Xylose was present either as 2,3,4-tri-*O*-methyl pentitol (terminal residues) or as 2,3-di-*O*-methyl pentitol [(1-4)-linked chains]. Under-methylation was detected by an excess of apparent branch points over terminal residues (11%:4%) and by the presence of unmethylated hexose.

The mannan was more easily methylated following reduction of the carboxyl groups and it then showed no signs of under-methylation (Table 6b). The main component was (1-3)-linked mannose (64%), with small amounts of terminal pentose (2.9%) and (1-4)-linked pentose (1.6%). A considerable increase in both terminal hexose (1–15%) and (1-2-3)-linked hexose (4–12%) was observed compared with the unreduced mannan. These data are consistent with the presence of glucuronic acid as terminal residues linked directly to carbon 2 of mannose units in the (1-3) backbone. Other linkages detected were (1-2-3)-linked branch points and a small amount of (1-3)-linked glucose. No trace of 2,4-di-*O*-methyl hexitol or 6-methyl hexitol was found in the reduced mannan.

Isolation of a polyuronic acid

Polysaccharides consisting mainly of uronic acids can be purified from mixtures containing neutral polysaccharides by procedures that exploit their resistance to acid hydrolysis (Tsuchihashi *et al.*, 1983). A sample (100 mg) of the alkali-soluble fraction of the wall was hydrolysed in 10 ml 0.2 M-trifluoroacetic acid for 24 h at 100 °C. The supernatant was discarded and the residue

Table 7. ^{13}C -NMR analysis of the polyglucuronan

The ^{13}C -NMR spectrum of the neutral-soluble fraction, peaks were assigned based on the spectrum of a polyglucuronic acid (Tsuchihashi *et al.*, 1983).

Peak	p.p.m.	Mucoric acid	Difference	Assignment*
1	174.6	175.7	-1.1	C6 (1-4) β -D GlcA
2	102.6	103.4	-1.0	C1 (1-4) β -D GlcA
3	80.8	82.0	-1.2	C4 (1-4) β -D GlcA
4	75.3	76.6	-1.3	C5 (1-4) β -D GlcA
5	74.3	75.5	-1.2	C3 (1-4) β -D GlcA
6	72.9	74.0	-1.1	C2 (1-4) β -D GlcA

*GlcA, Glucuronic acid.

was made up to 10 ml with water and neutralized with NaOH to yield soluble and insoluble residual fractions.

The soluble fraction was excluded from a Fractogel HW-40-S column which indicated that it has an M_r of 10000 or greater. The carbazole assay showed that it was 99% glucuronic acid.

The ^{13}C -NMR spectrum of the soluble fraction was recorded and the peaks assigned based on the spectrum of a polyglucuronic acid (Tsuchihashi *et al.*, 1983), (Table 7). The spectrum of the soluble fraction matched that of mucoric acid [poly β (1-4)-D-glucuronic acid],

although peaks were consistently shifted down-field by 1.0 to 1.3 p.p.m. The ^1H -NMR profile contained two peaks at 4.714 and 4.688 p.p.m., which were identified as belonging to the anomeric (C1) proton of D-glucuronic acid; the coupling constant of these two peaks indicates that the glucuronic acid units are joined by β -linkages. These data are also consistent with a structure similar to that of mucoric acid. This fraction represents at least 2.5% of the total wall or 25% of the wall uronic acid and is therefore likely to be a significant wall component.

Electron microscopy of *T. cutaneum* and *C. albicans*

Cells of *T. cutaneum* and *C. albicans* were grown for 40 h and 16 h, respectively on YPD medium, then fixed with 1.5% KMnO_4 and embedded in Epon 812 as described by Kreger-van Rij & Veenhuis (1971) except that cells were post-stained for 1 h with saturated uranyl acetate in 100% ethanol prior to embedding. Sections were further post-stained for 1 h with saturated uranyl acetate in 50% ethanol to enhance contrast. The walls of *T. cutaneum* showed a lamellar structure characteristic of a basidiomycetous yeast (Fig. 1). In contrast, the wall of ascomycetous yeasts such as *C. albicans* showed a thick electron-lucent layer of wall surrounded by a thin electron-dense layer of mannoprotein (data not shown).

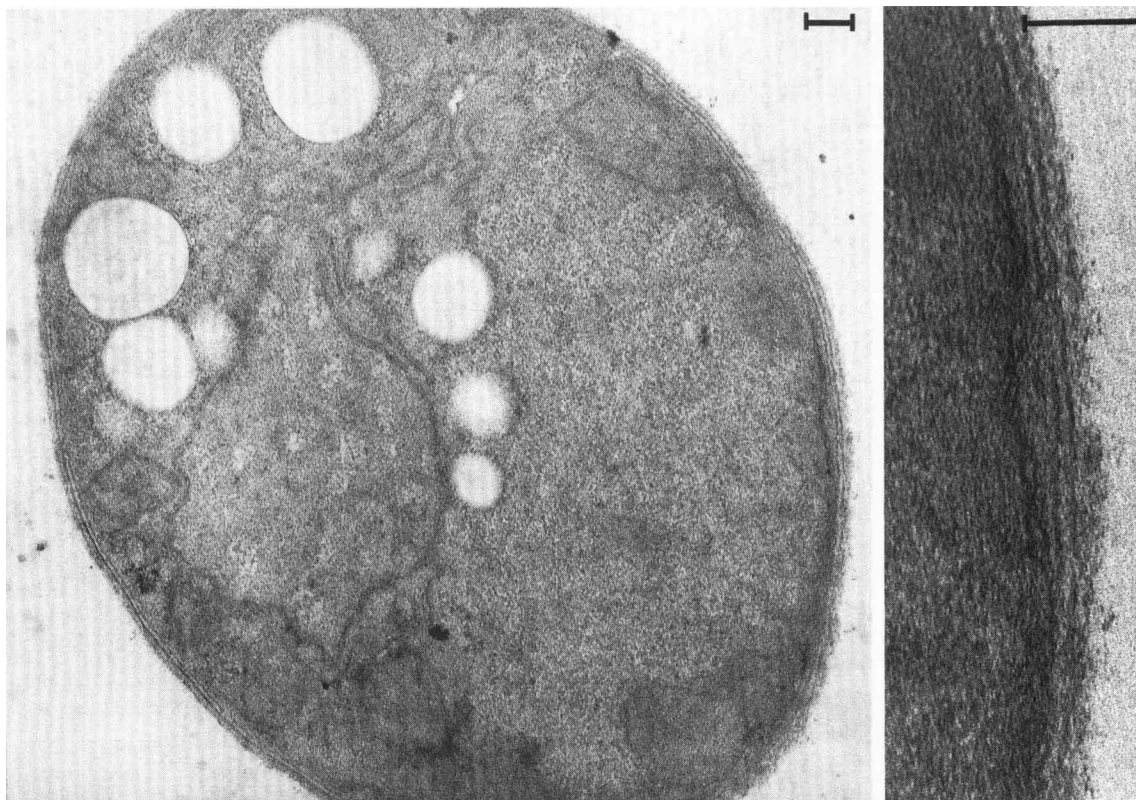


Fig. 1. A section of *T. cutaneum* prepared as described in Results. Bars, 0.2 μm .

Discussion

The proportions of alkali-soluble and -insoluble material in the wall preparations of *T. cutaneum* were similar to those found in the wall of *Saccharomyces cerevisiae* (Fleet, 1985) but the chemical composition of the wall and fractions differed markedly. Both chitin (9%) and neutral carbohydrate (63%), were present in amounts more characteristic of filamentous fungi such as *Schizophyllum commune* (Sietsma & Wessels, 1977) or *Trichoderma viride* (Benitez *et al.*, 1975) than of a yeast. Similarly the content of mannose in the wall of *T. cutaneum* (6%) was much lower than that found in *Saccharomyces cerevisiae* (25–50%; Fleet, 1985). Uronic acid accounted for 13% of the total wall of *T. cutaneum*. Cell walls of *T. cutaneum* have not previously been assayed for glucuronic acid, but it has been found (Weijman, 1979) in cell hydrolysates of *T. beigelii* (= *T. cutaneum*). Some species of yeast, such as the pathogen *Cryptococcus neoformans*, produce extracellular polysaccharides containing uronic acid but these are not structural components of the wall (Vartivarian *et al.*, 1989). As far as we are aware, the only other fungi possessing comparable amounts of uronic acid in their yeast or hyphal walls are the Zygomycetes such as *Mucor* spp. (Bartnicki-Garcia & Reyes, 1968) which are not related to *Trichosporon*.

Hydrolysates of the alkali-insoluble fraction contained glucose, glucosamine and small amounts (7%) of uronic acid. GLC-MS analysis of the methylated fraction showed that the glucose units were linked by a mixture of (1-3) and (1-6) bonds. The glucan in this fraction is likely to be β -linked as cells of *T. cutaneum* were lysed by either snail gut extract or by fractions of Novozyme 234 which did not contain detectable α glucanase activity (Depree, 1992). Exhaustive digestion of the alkali-insoluble fraction with Novozyme 234 released only *N*-acetyl glucosamine and no glucosamine, indicating that the wall contains chitin rather than chitosan. Exhaustive digestion of the alkali-insoluble fraction of *T. cutaneum* with Zymolyase released reducing sugar equal to at least 12% of the total dry wt, which indicates that this fraction contains β (1-3) glucan. The remaining glucan is probably protected from Zymolyase by the disposition of β (1-6) branch points along the β (1-3)-linked main chain.

The alkali-soluble fraction contained glucose, mannose, xylose, a small amount of galactose and most of the glucuronic acid. Methylation analysis revealed that 90% of the neutral carbohydrate in this fraction was (1-3)-linked hexose, including 66% glucose, 18% mannose and 5% galactose. Other bond types found in the alkali-soluble fraction included (1-4)-linked xylose, (1-3-4)-linked hexose, (1-2-3)-linked hexose and (1-3-6)-linked

hexose. Exhaustive digestion of this fraction with Zymolyase [a preparation containing β (1-3) glucanases; Kitamura & Yamamoto, 1972] did not cause a measurable release of reducing sugar, whereas Novozyme 234 [which contains both α and β (1-3) glucanases (Peberdy, 1979)] released reducing sugar equal to approximately 75% of the total glucose. These data suggest either that the alkali-soluble glucan is α (1-3)-linked, or that it is protected from β -glucanases.

Two mannan fractions with M_r of 400 000 or higher were isolated from a sample of the alkali-soluble fraction and purified by ion-exchange chromatography. Both fractions contained xylose but no galactose and were therefore pentosyl (xylo) mannans rather than galactomannans. This is consistent with the biochemical similarities between our isolate of *T. cutaneum* and other basidiomycetous species of *Trichosporon*, which have been shown to produce pentosyl mannans rather than galactomannans (Gorin & Spencer, 1968). Neither fraction contained significant amounts of arabinose, but unlike the extracellular polysaccharide of another yeast described as *T. cutaneum* (Gorin & Spencer, 1967), they contained no significant amounts of fucose, which is also a component of the galactomannan of *T. pullulans* (Gorin & Spencer, 1968). Insufficient material precluded a determination of the uronic acid content of these fractions.

An acidic mannan isolated from whole cells was found to have a molecular mass, profile on ion exchange and content of neutral sugars similar to the two fractions isolated from the alkali-soluble portion of the wall. The acidic mannan contained glucose, mannose, xylose and glucuronic acid in the proportions 1:10:2.5:3. The uronic acid component of this mannan was positively identified as D-glucuronic acid by carboxyl reduction and assay with glucose oxidase reagent. The acidic mannan lacks the α (1-6) mannose backbone found in the mannans of *C. albicans* and *Saccharomyces cerevisiae* (Ballou, 1982; Saxena *et al.*, 1989), but instead appears to have an α (1-3)-linked backbone to which single residues or short (1-4)-linked chains of xylose are attached. A comparison of the methylation patterns of the carboxyl-reduced mannan as compared to the unreduced material showed a considerable increase in both 2,3,4,6-tetra-*O*-methyl hexose (terminal residues) and 4,6-di-*O*-methyl hexitol [(1-2-3)-linked branch points]. The linkages of the glucose residues were not investigated, but it is noteworthy that recent studies (Van Rinsum *et al.*, 1991) have shown that mannoprotein of *Saccharomyces cerevisiae* contains covalently linked glucose residues.

The presence of uronic acid moieties in a carbohydrate interferes with permethylation analysis in two ways. First, uronic acid residues are not detected by GLC-MS as used in this study. Second, any neutral sugars attached

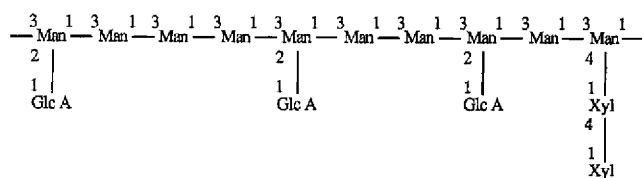


Fig. 2. Proposed structure of the carbohydrate component of the *T. cutaneum* acidic mannan.

to the uronic acid residues are not released by acid hydrolysis and thus are also not detected. The apparent increase in both terminal and (1-2-3)-linked branch point residues therefore is consistent with the presence of the glucuronic acid as single terminal residues linked directly to the backbone by (1-2-3) branch points. This structure (Fig. 2) is similar to the one proposed for the capsular polysaccharides of *Cryptococcus neoformans*, and in particular contains (1-4)-linked xylose and (1-2)-linked terminal glucuronic acid residues. These features are two of the main immunological markers for *Cryptococcus neoformans* capsular polysaccharide (Cherniak *et al.*, 1980) and this structural similarity would be in keeping with the immuno cross-reactivity observed between the capsular antigen of *Cryptococcus neoformans* and a cellular antigen of *T. beigeli* (McManus *et al.*, 1985).

A poly β (1-4) glucuronan analogous in structure to a polymer called mucoric acid (Tsuchihashi *et al.*, 1983) was isolated after prolonged acid hydrolysis of the alkali-soluble fraction. It consisted of chains of 50 or more glucuronic acid residues and represented 42% of the total glucuronic acid in the alkali-soluble fraction. Since this glucuronan was highly soluble in water, but could not be extracted by a simple hot-water extraction procedure, it follows that it is a component of a much larger, insoluble polymer. It is possible that the poly-glucuronide is a component of an acidic glucan such as those isolated from the fruiting bodies of *Polyporus fomentarius* and *Polyporus igniarius* (Björndahl & Lindberg, 1970).

The glucans isolated from *Polyporus* spp. contain an average of 4-5 β (1-4)-linked glucuronic acid residues attached to a β (1-3)-linked backbone by (1-3-6)-linked branch points (Björndahl & Lindberg, 1970). If an acidic glucan analogous to those isolated from *Polyporus* were analysed by methylation and GLC-MS under the conditions specified in this study, the (1-3-6)-linked branch points would not be detected as they would remain attached to the glucuronan side chains. The methylation profile obtained would therefore appear to be of a predominantly (1-3)-linked glucan with very few (1-6)-linkages, similar to the profile obtained for the *T. cutaneum* alkali-soluble glucan. A structure of this type may also be resistant to β (1-3) glucanases such as Zymolyase.

The chemical composition of the *T. cutaneum* cell wall gives some clue as to the proportions and nature of the types of polymers present. The main component of the wall is glucan (30-40%) followed by chitin (9%) and glucuronan (13%). Mannan (5%) is a minor but significant component of the wall.

Until now the biochemistry of oil production and lactose utilization by *T. cutaneum* had been studied in considerable detail but little was known of the basic biology of this yeast. We have shown that the wall of *T. cutaneum* differs markedly from that of *Saccharomyces cerevisiae* or *C. albicans*, but has some similarities with the wall of filamentous fungi. A lamellar structure characteristic of the wall of Basidiomycetes (Kreger-van Rij & Veenhuis, 1971) has been observed in this isolate (Fig. 1). The production of an acidic xylomannan rather than a galactomannan by this organism is consistent with its classification as a basidiomycetous species of the genus *Trichosporon* (Gorin & Spencer, 1968). The similarities between the acidic mannan of *T. cutaneum* and similar acidic mannans of *Cryptococcus neoformans* and *Tremella mesenterica* suggest a possible evolutionary relationship between these organisms.

As far as we are aware, this is the first time that a β -glucuronan has been isolated from a yeast not belonging to the Zygomyceteaceae. It is possible therefore that this type of polymer may be found among other genera. Preliminary data (Depree, 1992) suggests that these polyuronic acid chains play a major role in contributing to wall structure through the formation of ionic bonds.

Although *T. cutaneum* is usually referred to as an imperfect yeast, it is useful to remember that this organism also grows in a filamentous form; particularly in view of the general similarity between the walls of *T. cutaneum* and those of various filamentous fungi. The organism was first isolated from the floor drain of a dairy factory (Moon *et al.*, 1978). It seems likely that it is found in nature as a filamentous fungus growing in soil and leaf litter, and only assumes the yeast form when grown on whey or other liquid media.

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