COMPARATIVE QUALITATIVE ANALYSES OF HYDROLYSIS PRODUCTS OF EXTRACELLULAR POLYSACCHARIDES PRODUCED BY SOME YEASTS AND YEAST-LIKE FUNGI

by

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ABSTRACT

The objective of the experiments was to compare qualitatively the monosaccharides in the hydrolysis products of the extracellular polysaccharides of several yeasts and yeast-like fungi. Specifically, the study was aimed at finding similarities and differences that might be useful in suggesting and supporting taxonomic relationships. Gas chromatography and paper chromatography were used as methods of analyses in an effort to find out what method is sufficient at the qualitative level for distinguishing some genera of yeasts and yeast-like fungi; and what method would be best at the quantitative level for distinguishing amongst some species of the same genus. From the analytical results it was found that paper chromatography using the solvents ethyl acetate: pyridine: water, (8:2:2) was sufficient for qualitative determination of the monosaccharides in the extracellular polysaccharide hydrolysis products. However, indications were that quantitative analyses by gas chromatography, using the trimethylsilyl derivatives of the monosaccharides would have been successful in distinguishing among species of the same genus. Two groups were formed on the bases of the qualitative results.

Group I contained two subgroups. Subgroup I encompassed those yeasts and yeast-like fungi with the monosaccharides galactose, glucose, mannose, xylose present in the hydrolysis products of their extracellular polysaccharides. Included in this Subgroup I are: Cryptococcus laurentii, Tremella mesenterica, Bullera alba, Sporobolomyces odorus, Sporobolomyces singularis, and Rhodotorula glutinis. Subgroup II is Ustilago hordei only, with the monosaccharides galactose, glucose, mannose, and lacking xylose. Group II contains Taphrina populina only, with glucose and mannose present and both galactose and xylose absent.

The two groups formed support some of the taxonomic relationships that have already been suggested. The Tremella - Cryptococcus taxonomic relationship that had previously been postulated on the basis of similarities in extracellular polysaccharide hydrolysis products, morphology, carbon assimilation patterns, enzymatic xylosylation reaction, and starch formation was supported. Secondly, the Cryptococcus-Bullera relationship that had been suggested on the basis of inositol assimilation, lack of pseudomycelium, and similarities in starch synthesis, was supported by the qualitative analysis of the monosaccharides present in the extracellular polysaccharide hydrolysis products. The

monosaccharides found in both Cryptococcus laurentii and Bullera alba extracellular polysaccharides were the same qualitatively. Duality amongst species of Sporobolomyces might be supported with further work using quantitative gas chromatographic analyses. This duality had been postulated on account of the duality shown in antigenic analyses and percent G+C base analyses of DNA. Taphrina populina can be distinguished from Rhodotorula glutinis and Cryptococcus laurentii. Cryptococcus laurentii produces starch and assimilates inositol: Rhodotorula glutinis assimilates inositol but does not produce starch; and Taphrina populina produces starch but does not assimilate inositol. Two monosaccharides present in the extracellular polysaccharide hydrolysis products of both Cryptococcus laurentii and Rhodotorula glutinis are galactose and xylose whereas Taphrina populina lacks these two monosaccharides.

Results obtained from the qualitative analyses of the extracellular polysaccharides produced by fungi may be

important taxonomically. This is because the qualitative information may be used when deciding on Perfect-Imperfect fungal relationships. However, this information should be considered along with data from other fields such as morphology, cytology, and genetics before hypothesizing on a taxonomic relationship.

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INTRODUCTION

In general, the purpose of the experiments was to gather valuable information that may aid in the synthesis of a classification scheme. The data obtained from the qualitative analyses of the extracellular polysaccharides of some yeasts and yeast-like fungi may give support to some of the already postulated Perfect-Imperfect fungal relationships. This process of critically re-examining, adding to, and correlating the results of previous workers is a necessary part of any research.

Specifically, the objectives of the experiments were:

1. To determine qualitatively the hydrolysis products of the extracellular polysaccharides produced by several yeasts and yeast-like fungi.
2. To employ both gas chromatography and paper chromatography as methods of analysis and to determine:
   (a) which method is best at the qualitative level,
   (b) which method is best for separating genera only, and species of the same genus.
3. To compare and contrast the qualitative results with those of previous workers.

4. To compare and contrast the yeasts and yeast-like fungi investigated with respect to the monosaccharides in their extracellular polysaccharide hydrolysis products.
5. To arrange the yeasts and yeast-like fungi investigated into groups on the basis of qualitative similarities or differences in the monosaccharides in their extracellular polysaccharide hydrolysis products.
6. To suggest taxonomic relationships considering the above formed groups in conjunction with data supplied from other fields such as morphology and genetics.
7. To examine the validity of using certain biochemical analyses, in particular, those of the extracellular polysaccharides in the synthesis of a classification scheme.

LITERATURE REVIEW

For convenience, the yeasts and yeast-like fungi used in the experiments will be reviewed in groups. Group I Subgroup I contains Tremella mesenterica Fr., Cryptococcus laurentii (Kufferath) Skinner, Bullera alba (Hanna) Derx., Sporobolomyces singularis Phaff et Do Carmo Sousa, Sporobolomyces odorus Derx., and Rhodotorula glutinis (Fres.) Harrison. Group I Subgroup II is Ustilago hordei (Pers.) Lagerh. Group II is Taphrina populina Fr.

Group I Subgroup I

The extracellular polysaccharides of Tremella mesenterica and Cryptococcus laurentii have been investigated extensively. The polymer produced by Tremella mesenterica contains both an acidic heteropolysaccharide and a neutral glucan. Slodki, in 1966 (43), identified the acid hydrolysis products of the acidic heteropolysaccharide. These products were O-acetyl, xylose, mannose, and glucuronic acid in the ratio of 0.5 : 4.4 : 3.8 : 1 respectively. Fraser and Jennings, in 1971 (19), identified the neutral portion of the extracellular polysaccharide. It was a neutral glucan, linear in structure, composed of 200 α-D-glucopyranose units linked α1-6 and α1-4 in the ratio of 1:2 respectively.

Similarly, the extracellular polysaccharide produced by Cryptococcus laurentii contains an acidic and a neutral

fraction. Abercrombie et al, in 1960 (1), found the acidic fraction to be composed of D-Mannose, D-Xylose and D-Glucuronic acid. However, studies by Jeanes et al, in 1964 (24) indicated the presence of D-Mannose, D-Xylose and D-Glucuronic acid. O-Acetyl was determined to be seven percent. They also found some traces of galactose and glucose. Structural studies showed that the "backbone" was composed of D-Mannose and the end groups were D-Xylose and D-Glucuronic acid. The neutral portion of the extracellular polysaccharide contained D-Glucose linked 1-3, 1-4, 1-2 and/or 1-6 (24, 47). The biosyntheses of the carbohydrates found in the acidic fraction were also investigated by Abercrombie et al, 1960 (2). D-Mannose and D-Glucuronic acid were formed from the hexoses without any appreciable breakdown of the hexose skeleton. D-Xylose was formed from the hexoses mainly by a process involving the loss of carbon six; and D-Xylose and L-Arabinose were both converted to D-Mannose, D-Xylose, and D-Glucuronic acid with rearrangement of the pentose skeleton that may have involved the action of transaldolases and transketolases. The conditions for maximum production of the extracellular polysaccharides of Cryptococcus laurentii for industrial purposes were investigated by Cadmus et al, 1962 (12).

The literature suggests that evidence exists for

postulating first, in general, Cryptococcus-Basidiomycetous relationships and secondly, more specifically, Cryptococcus-Tremella relationships. First, the Cryptococcus-Basidiomycetous relationships were formulated on the basis of DNA base analyses, Storck, in 1966 (48), found the percent C+G content in some Basidiomycetes to be fifty percent. He also determined Cryptococcus albidus G+C percent to be fifty-five. Nakase and Komagata, in 1968 (33), found that five Cryptococcus species out of one hundred and forty yeasts tested had a G+C content of forty-six to fifty-six percent.

Second, the Cryptococcus-Tremella relationships were brought to light by several workers (28, 41). Slodki et al, in 1966 (41), proposed a possible taxonomic relationship because of similarities between some members of Cryptococcus and Tremella species. It was found that species of Tremella that produced polymers similar to those produced by Cryptococcus also had similar carbon assimilation patterns. All strains listed by Slodki et al (41) assimilated glucose, xylose, D-Arabinose, mannitol, adonitol, and trehalose. Both genera have the ability to symthesize starch at a pH of 5.0 or lower (32, 41).

The ready removal by acid hydrolysis of the xylose

residues, perhaps owing to their apparent peripheral location relative to the mannose-glucuronic acid "backbone" (1, 24) has led to the preparation of a useful acceptor for the study of enzymatic xylosylation reactions (13). Cryptococcus is a source of enzymes catalyzing the biosynthesis of xylosyl donor nucleotide, UDP-xylose (3). Extracts from Cryptococcus laurentii and Tremella mesenterica are nonspecific with respect to catalysis of xylosyl transfer from UDP-xylose to partially dexylosylated acceptor polymers belonging to either organism (41).

Under certain conditions basidiospores of some Tremellales bud to produce yeast-like colonies in culture that are similar to those of Cryptococcus (28).

Fraser and Jennings present evidence against the Cryptococcus-Tremella relationships (19). The neutral glucan of Tremella mesenterica has not been shown to contain the α1-3 linkages that are present in the Cryptococcus laurentii neutral glucan (1, 47).

The Tremella mesenterica neutral glucan is said to resemble structurally the neutral glucan produced by Pullularia species. The reasons for the resemblance are the α1-4, α1-6 linked glucopyranose units in both (8, 9, 11, 19, 35, 45, 53, 54).

Some species of Cryptococcus and Bullera could be taxonomically related (32). They assimilate inositol and break the α-glucosidic bonds of melibiose, melezitose, and methyl α-D-glucoside. Among others, they assimilate sucrose, lactose, cellobiose, and several pentoses. Starch synthesis is present and pseudomycelium is absent. Some strains of Bullera that have lost their ability to produce and discharge ballistospores might be classed with the genus Cryptococcus (32).

The extracellular polysaccharides of Rhodotorula glutinis, Sporobolomyces singularis, and Sporobolomyces species have also been investigated. Gorin et al (20), described the extracellular polysaccharide of Rhodotorula glutinis as a straight chain mannan, composed of at least ninety units of alternately linked β 1-3, β 1-4 D-mannopyranose residues. They also reported a hexose and a methyl pentose among hydrolysis products.

Slodki, in 1966 (43), reported the extracellular polysaccharide of two unidentified Sporobolomyces species to be a phosphorylated galactan of α 1-3, α 1-6 linked units in approximately equal proportions. D-Galactose and

D-Galactose-6-phosphate were the only components found on hydrolysis.

Gorin et al (21) stated that Sporobolomyces singularis produced a trisaccharide, galactosyl-lactose and a tetrasaccaride, galactobiosyl lactose from lactose. The D-Galactopyranosyl units were β 1-4 linked. Phaff and Spencer, in 1969 (36) reported an extracellular mannan from Sporobolomyces roseus and Sporobolomyces singularis that was similar to the extracellular mannan reported by Gorin et al (20) to be produced by Rhodotorula glutinis.

Several characteristics of Rhodotorula and Sporobolomyces species set them apart from the majority of yeasts. Antigenic analyses by Tsuchiya et al (51) indicated that species of Rhodotorula and Sporobolomyces had no common antigen. Also five groups were synthesized that did not give cross reactions with each other. Rhodotorula and Sporobolomyces species were not contained in the group that consisted of the majority of yeasts. The cell walls of Sporobolomyces and Rhodotorula species seem to be similar in composition yet different from most other yeasts. They are said to have a low glucose content, (or lack it completely) and a high chitin content. A mannan that is capable of giving a precipitate with Fehling's solution is absent (15, 30, 46).

The relationships of Rhodotorula and Sporobolomyces species to the Basidiomycetes has been put forward by several workers. Storck, in 1966 (48), working on DNA base analyses found a duality in both the Sporobolomyces and the Rhodotorula species. This duality was reflected in the antigenic work of Tsuchiya mentioned previously (51).

Sporobolomyces roseus was found to have a C+G ratio of fifty percent and Sporobolomyces salmonicolor and Rhodotorula mucilaginosa both had a C+G ratio of sixty-five percent. All three of these C+G readings are within the range expressed for Basidiomycetes. Nakase and Komagata, in 1968 (33), found that species with strong urease activity had a high G+C content, implying a Basidiomycetous relationship. In general, eleven Rhodotorula species out of one hundred forty species of yeasts tested had 47.5 to 65 percent G+C content.

Further information on the relationship of Rhodotorula and Sporobolomyces species to the Basidiomycetes is concerned with their life cycles. Rhodotorula glutinis was demonstrated to be the imperfect stage of the Ustilagenaceous Rhodosporidium toruloides by Banno, in 1967 (4). Newell and Fell, in 1970, (34), found that the haploid mating type strains of Rhodosporidium sphaerocarpum were identical with strains of

Rhodotorula glutinis. Lodder and Kreger van Rij (31) and Lodder et al (14) suggested that the genus Rhodotorula is an imperfect or degenerate Basidiomycete. They stated that some of the species of the genus Rhodotorula, were to be considered asporogenous Sporobolomyces species, since Sporobolomyces without ballistospore production would be indistinguishable from Rhodotorula species. Kluyver and van Niel (27) first raised the possibility that the genus Sporobolomyces might be of Basidiomycetous origin. This was because the ejaculation mechanism of the ballistospores of Sporobolomyces is the same as the ejaculation mechanism of basidiospores.

van der Walt and Pitout (52) submitted evidence based on the DNA analysis for the existence of 2N (diploid) and N (haploid) generations of Sporobolomyces salmonicolor. However, because the ballistospores of diploid colonies produce only a diplophase and the ballistospores of haploid colonies produce only a haplophase, the possibility of the ballistospores being basidiospores (meiospores) as suggested by Sainclivier (39, 40) is ruled out. Banno, 1967 (4), found no conjugation between Rhodotorula and Sporobolomyces strains investigated, and he therefore stated that there was no possible relationship between ballistospore production by

Sporobolomyces and the sexual cycle concerned with Rhodotorula.

Group I Subgroup II

The extracellular polysaccharide of Ustilago hordei has not been investigated.

Group II

Morphologically, cultures of the yeast stage of Taphrina populina were said to resemble Cryptococcus and Rhodotorula (32). However, Taphrina does not assimilate inositol, therefore distinguishing it from Cryptococcus; it does produce starch which distinguishes it from Rhodotorula.

Wickerham, in 1952 (55) suggested a relationship between Lipomyces and Taphrina based on starch production and multispored asci found in both genera. However, Kramer, in 1960 (29) found that the ascus development in Taphrina is altogether different from Lipomyces. This fact would make any relationship between Taphrina and Lipomyces doubtful.

MATERIALS AND METHODS

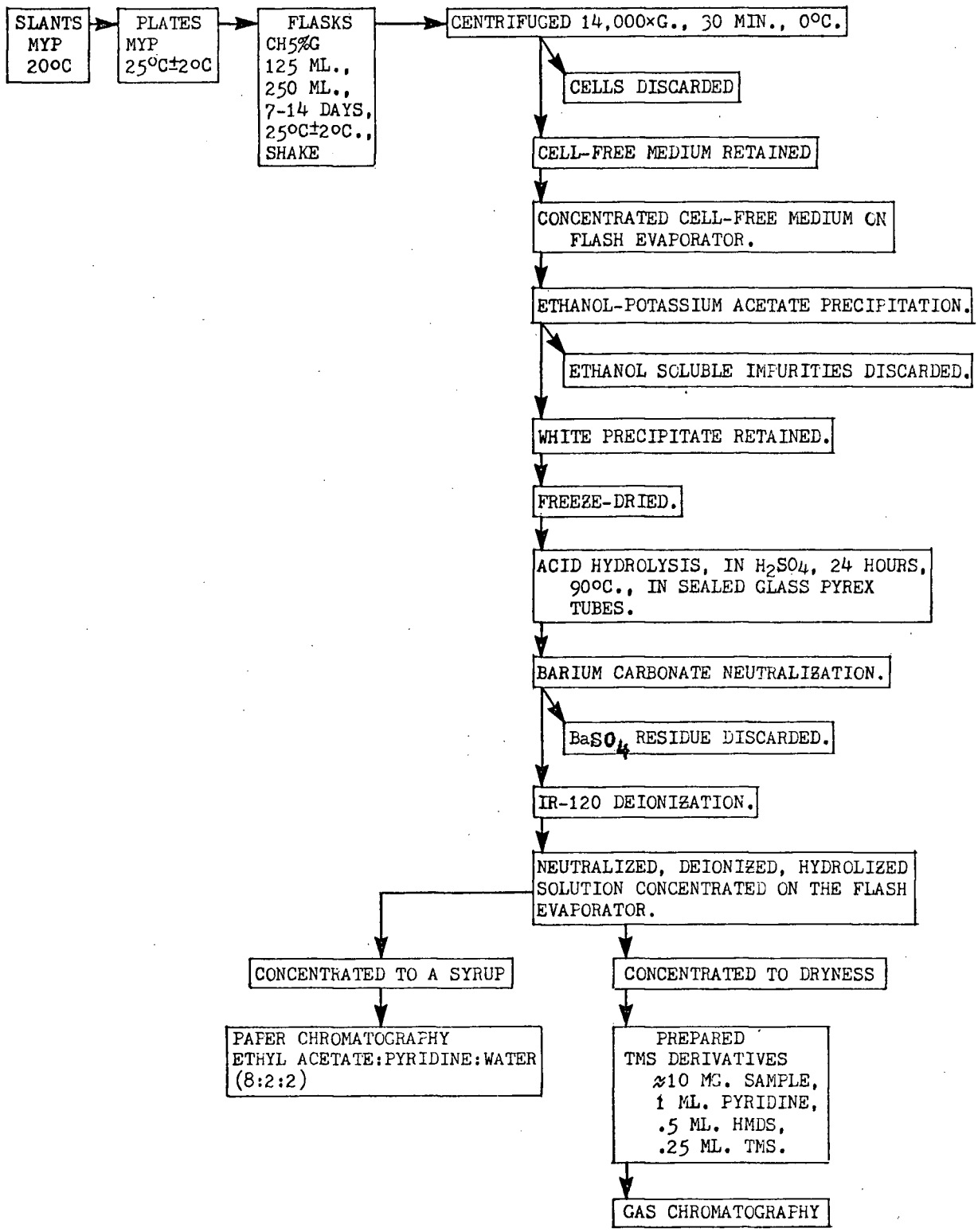
For convenience, Materials and Methods are discussed in three sections. Section A covers maintenance of cultures for polysaccharide production, Section B deals with the isolation of the extracellular polysaccharide and Section C deals with the analysis of the crude extracellular polysaccharide.

Fungi were obtained from the Mycology Laboratory, Department of Botany, University of British Columbia, Vancouver, B. C.

Fungus	Host	Location	Collection Date	Collector
Cryptococcus laurentii NRRLYB-4920 (U.B.C.#8114)				
Bullera alba (Hanna) Derx. U.B.C. #983		Goldstream Provincial Park, B. C.	18/4/60	R.J.Bandoni
Sporobolomyces odorus U.B.C. #949	soil	Harrapa, West Pakistan	2/69	M.Rafiq
Sporobolomyces odorus. Derx. U.B.C. #981	Smut infected *Citrus* leaves	Nice, France	1930	H.G.Derx
Sporobolomyces singularis Phaff et Do Carmo-Sousa U.B.C. #8018	insect frass from dead *Tsuga*.	Oregon, U.S.A.	1962	
Rhodotorula glutinis U.B.C. #940 (I.F.O.#0559)				
Tremella mesenterica R.J.B.#2259-6	on fallen *Alnus*	U.B.C. Endowment Lands	14/10/61	R.J.Bandoni
Ustilago hordei U.B.C. #570 (C.Person E3(-))	*Hordeum*		1964	P.L.Thomas
Taphrina populina U.B.C. #249	*Poplus* leaf	U.B.C. Endowment Lands	7/68	S.Reid

Figure 1

Procedure for the production, isolation, and analysis of the extracellular polysaccharide.


SLANTS
MYP
20°C
PLATES
MYP
25°C±2°C
FLASKS
CH5%G
125 ML.,
250 ML.,
7-14 DAYS,
25°C±2°C.,
SHAKE
CENTRIFUGED 14,000×G., 30 MIN., 0°C.
CELLS DISCARDED
CELL-FREE MEDIUM RETAINED
CONCENTRATED CELL-FREE MEDIUM ON FLASH EVAPORATOR.
ETHANOL-POTASSIUM ACETATE PRECIPITATION.
ETHANOL SOLUBLE IMPURITIES DISCARDED.
WHITE PRECIPITATE RETAINED.
FREEZE-DRIED.
ACID HYDROLYSIS, IN H_2SO_4, 24 HOURS, 90°C., IN SEALED GLASS PYREX TUBES.
BARIUM CARBONATE NEUTRALIZATION.
$BaSO_4$ RESIDUE DISCARDED.
IR-120 DEIONIZATION.
NEUTRALIZED, DEIONIZED, HYDROLIZED SOLUTION CONCENTRATED ON THE FLASH EVAPORATOR.
CONCENTRATED TO A SYRUP
PAPER CHROMATOGRAPHY
ETHYL ACETATE:PYRIDINE:WATER
(8:2:2)
CONCENTRATED TO DRYNESS
PREPARED
TMS DERIVATIVES
≈10 MG. SAMPLE,
1 ML. PYRIDINE,
.5 ML. HMDS,
.25 ML. TMS.
GAS CHROMATOGRAPHY

Section A

Maintenance of Cultures for Polysaccharide Production

Fungi were maintained on MYP medium in culture tubes at 20°C. The fungi were transferred with a small sterile (flamed and cooled) wire loop from the MYP tube cultures to MYP plates. Contaminants were revealed by microscopic examination of the cultures. Tetracycline was used to combat bacteria.

MYP Medium used for tubes and plates.

1. Ingredients:

Malt Extract	15 grams	Bacto Malt Extract, Difco Laboratories
Yeast Extract	0.5 grams	Bacto Yeast Extract, Difco Laboratories
Soytone or Peptone	2.5 grams	Bacto Peptone, Bacto Soytone, Difco Laboratories
Distilled water	1,000 millilitres	
Agar	15 grams	
Tetracycline	8 ml./litre of medium as required.	Nutritional Biochemical

2. Preparation of the Medium

Plates: The medium was autoclaved for twenty minutes at

15 psi., cooled, poured, solidified. Plates were inoculated as described above, and unused plates were stored in the refrigerator.

Tubes: Slants were prepared by filling tubes with the required amount of molten agar. The agar was allowed to solidify with the tube resting at an angle. Unused slants were stored in the refrigerator. The tubes were inoculated as described above.

3. Build up of inoculum

Pure cultures on MYP agar were used to build up a large amount of inoculum. Cultures were cut lengthwise or scraped from the agar, and transferred to liquid Casein Hydrolysate 5% Glucose Medium (CH5%G). Liquid CH5%G was increased gradually as the fungal inoculum built up. Liquid cultures were shaken reciprocally at 25°C ± 2°C for seven to fourteen days.

4. Casein Hydrolysate 5% Glucose Medium (1, 19)
(for ten 2,800 ml. flasks)

Ingredients

Medium I

Distilled water	4 litres	
Casein Hydrolysate	15 grams	- Vitamin Free, Salt Free, Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

KNO_3	5 grams
KH_2PO_4	20 grams
K_2HPO_4	15 grams
$MgSO_4 \cdot 7H_2O$	2.5 grams
Thiamine Hydrochloride	5 milligrams
Trace Elements Solution	25 millilitres

Ingredients

Trace Elements Solution	$ZnSO_4 \cdot 7H_2O$	2.0 grams
	$CuSO_4 \cdot 5H_2O$	0.1 grams
	$MnSO_4 \cdot H_2O$	1.5 grams
	$FeSO_4 \cdot 7H_2O$	2.0 grams
	Distilled Water	1 litre

Ingredients

Medium II

Distilled water	1 litre
Glucose	250 grams
Tetracycline	8 ml. / litre as necessary

5. Preparation and Inoculation of CH5%G Medium

Medium I was divided among ten conical 2,800 ml. flasks. Flask tops were covered with foil. Media I and II were autoclaved for twenty minutes at 15 psi. Under sterile conditions, 100 ml. aliquots of Medium II were added to each of the ten flasks. Each flask was inoculated with a

suspension of yeast and sterile distilled water. Liquid cultures were shaken on a reciprocal shaker at 25°C ± 2°C for seven to fourteen days.

Casein Hydrolysate 5% Glucose Medium was chosen because it was used for polymer production by both Cryptococcus laurentii (1), and Tremella mesenterica (19) and because it was more chemically defined than either MY Broth with 5% Glucose (22, 41, 44) or the Autolyzed Brewer's Yeast Medium with 5% Glucose (12, 24).

Section B

Extraction and Isolation of the Extracellular Polysaccharides

1. Centrifugation of the liquid culture and recovery of the cell free medium.

After two weeks growth, the liquid culture was centrifuged at 14,000 g., for thirty minutes, at zero degrees centigrade. The cell free medium was retained and the cells were discarded. Samples of the cell free medium were checked microscopically to make sure that all the cells had been removed during centrifugation.

2. Ethanol-Potassium Acetate precipitation of the crude extracellular polysaccharide from the cell free medium.

A small sample of the cell free medium was concentrated to about one-half its original volume of the flash evaporator. A measured amount of the concentrated medium was added slowly, with shaking, to a known quantity of cold ethanol. Small amounts of potassium acetate were added to facilitate the precipitation of the polysaccharide. When a white preceipitation appeared the quantity of concentrated medium that had been added to the known quantity of ethanol was recorded. The precipitation was then carried out on a large scale. Dry ice was added to the ethanol to agitate

and keep it cold during the precipitation. The fibrous white precipitate was retained by centrifuging or decanting off the ethanol and ethanol soluble impurities. The white precipitate was dissolved in a small amount of distilled water and the resulting aqueous solution was added to ethanol as before. The white precipitate was centrifuged down, retained, then dissolved in a small amount of distilled water. The crude extracellular polysaccharide was poured into several round bottom flasks and freeze-dried overnight. The crude, fluffy, white, freeze-dried product was weighed and bottled. Parafilm was used to keep moisture out of the bottles.

3. Hydrolysis of the crude extracellular polysaccharide.

A portion of the freeze-dried crude extracellular polysaccharide was hydrolyzed with IN H_2SO_4 for twenty-four hours at 100°C in sealed glass Pyrex tubes.

4. Neutralization with Barium Carbonate and deionization with IR-120 cation exchange resin.

(a) Neutralization with $BaCO_3$

A slurry of barium carbonate and distilled water was prepared in a beaker in the fume hood. The slurry was added, a small amount at a time, to a flask containing the

solution of acid hydrolyzed material. The flask was swirled vigorously and neutralization was determined with litmus paper. The resulting mixture was filtered through a funnel; the cleared, neutralized solution was retained and the $BaSO_4$ residue was discarded.

(b) Preparation of the IR-120 cation exchange resin column

Regeneration of IR-120 cation exchange resin.

The resin was placed in a beaker at least four times the volume of the resin. The resin was washed several times with water, decanting all the water off each time. Enough 2N NaOH was added to the resin so that a basic reaction on litmus paper was obtained after the resin and 2N NaOH had been well stirred and left for at least ten minutes. The resin was washed completely free of NaOH. 2N HCl was added to the resin until a very positive acid pH was reached. The resin and the 2N HCl were stirred very well. The resin was then washed relatively free of acid. The acid treatment was repeated and the resin was then washed several times with water. The washed resin was transferred to a glass column and washed with water until a negative chloride test was obtained (16).

Preparation of the IR-120 column for deionization of the $BaCO_3$ neutralized polysaccharide

A glass column of slurried IR-120 was prepared as in

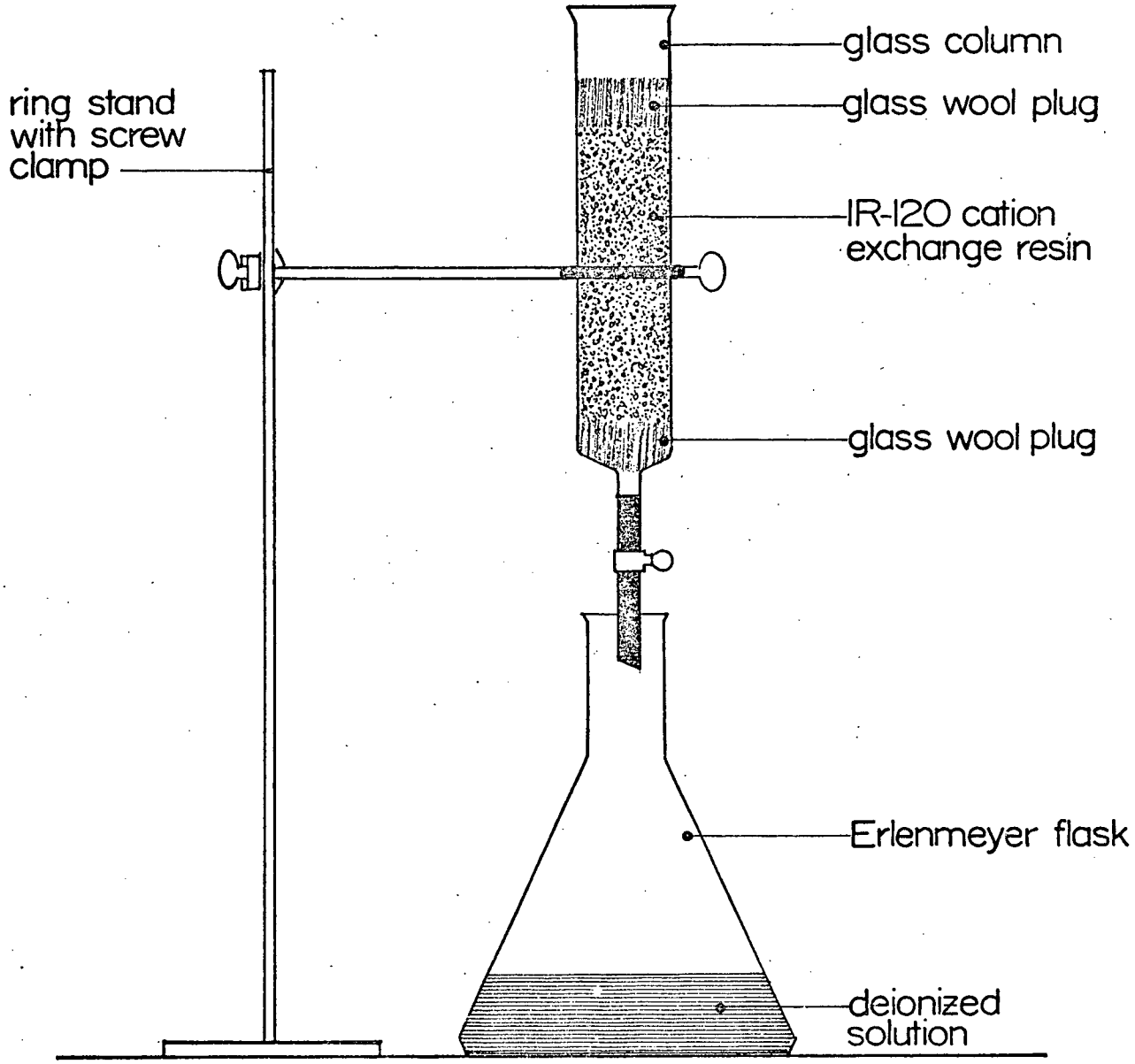
Figure 2. A plug of glass wool was inserted using a glass rod at the bottom of the column. The required amount of IR-120 cation exchange resin was slurried with distilled water and poured into the column along a glass rod. The rod was removed and the resin allowed to settle. Another small glass wool plug was inserted at the top of the column of IR-120. A piece of rubber tubing and a screw clamp were attached to the bottom of the glass column. The column was washed several times with distilled water, then the neutralized solution of hydrolysis products was poured into the column. This material was washed through with distilled water till the Molisch test for carbohydrate material was negative. An Erlenmeyer flask was used to collect the deionized solution of neutralized hydrolysis products.

The Molisch test for Carbohydrates (18, 26, 56)

In general, the Molisch test is the action of strong sulphuric acid on sugars and subsequent reaction of the products formed with phenolic substances. Specifically, in this test, if performed in a test tube: to approximately 0.05 grams of carbohydrate in 1 ml. of water was added one to two drops of fifteen percent alcohol solution of naphthol. Concentrated sulphuric acid was added by pouring it slowly down the side of the test tube to form a layer under the sugar

Figure 2

Apparatus for the IR-120 cation exchange resin.


glass column
glass wool plug
IR-120 cation exchange resin
glass wool plug
ring stand with screw clamp
Erlenmeyer flask
deionized solution

solution. In the presence of carbohydrate material a violet color appeared at the interface of the two liquids because of the formation of furfural derivatives and their reaction with naphthol (18). It is believed (26) that the mechanism of the reaction proceeds under the influence of 15M sulphuric acid from the glucopyranose form to the straight chain aldehyde form with subsequent dehydration and ring closure to 5-hydroxymethylfurfural. This is followed by the hydrolytic scission of the 5-hydroxylmethyl group with the production of formaldehyde and furfural, furfural degradation products, or furfural polymers.

Important Properties of Amberlite IR-120.

The cation exchange resin is strongly acidic, sulfonated, polystyrene type of medium porosity. The apparent density (average) is 0.77g/ml.; the mesh size (wet) is 20-50 mesh; the void volume, 35-40%; degree of regeneration is 98% (minimum). Moisture holding capacity is 49-55% and the total exchange capacity is, by volume, 1.75 meq./ml. min. and by weight (dry), 5.0 meq./g.min.

Section C

Qualitative Analysis of the Crude Extracellular Polysaccharide Hydrolysis Products

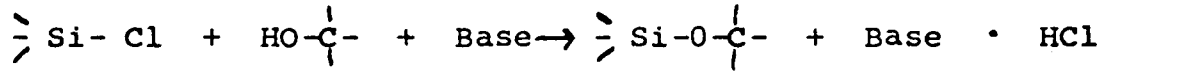
1. Gas Chromatography
2. Paper Chromatography

1. Gas Chromatography

Preparation of the trimethylsilyl derivatives of the neutralized, deionized hydrolysis products.

A portion of the neutralized, deionized, hydrolyzed extracellular polysaccharide was evaporated to dryness in a small round bottom flask on the flash evaporator. The preparation of the TMS derivatives was carried out in the fume hood. To approximately 10 mg. of sample was added 1 ml. of anhydrous pyridine (kept over KOH pellets). To the solution or suspension of material in pyridine was added 0.5 ml. HMDS (hexamethyldisilazane), and 0.25 ml. of TMS (trimethylchlorosilane). The solution became cloudy on the addition of TMS, presumably because of the precipitate ammonium chloride. The flask was stoppered immediately with a ground glass stopper or a covered cork and shaken vigorously for about thirty seconds, then allowed to stand for 5 minutes at room temperature. Formation of the TMS derivatives

occurred rapidly at room temperature. All free hydroxyl groups were silylated and the yield of the TMS derivatives was quantitative (49). In general, the silylation reaction as presented by Henglein and Scheinost (23) is:



The silylated material was injected with a glass syringe into the injector port of the gas chromatography machine. Chromatography was carried out on an F and M 720 dual column instrument. The two columns were 8 ft. x 0.25 in. coiled copper columns packed with equal weights (to within 20 mg.) of 20% SF 96 on 60-80 mesh Diatoport S. The columns were held isothermally at 190° for approximately three minutes and then programmed at 2° per minute to hold at 220°. Helium flow was approximately 88 ml. per minute (6.8 seconds for 10 ml.).

2. Paper Chromatography

A portion of the hydrolyzed, neutralized and deionized polysaccharide was flash evaporated to a syrup. Small amounts of the diluted (H_2O) syrup were applied with a flamed, cooled, wire loop to 24 in. by 7.4 in. Whatman #1 Chromatography Paper. At the same time, spots were also made of known monosaccharides. Descending paper chromatography was carried out using the solvents ethyl acetate: pyridine:

water in the ratios of 8:2:2, in an equilibrated glass chromatography tank for approximately 48 hours. The chromatograms were removed from the tank, dried, then developed with $AgNO_3$ dip (50).

Preparation of the $AgNO_3$ dip

The principle behind the $AgNO_3$ dip process is based on the Tollen's silver mirror test for carbohydrates. The silver is reduced by aldehydes and sugars containing free aldehyde or ketone groups (8).

Ingredients (50)

1. Acetone-$AgNO_3$ Solution

This reagent solution was prepared by diluting 0.1 ml. of saturated aqueous silver nitrate solution to 20 ml. with acetone, and adding water dropwise, with shaking, until the silver nitrate which separates on the addition of acetone has redissolved. Spreading of the spots is limited because of the sparing solubility of sugars in acetone. (0.014% at 23°C for crystalline glucose.)

2. Ethanol - NaOH Solution

The 0.5N solution of NaOH in aqueous ethanol was made by diluting saturated aqueous NaOH solution with ethanol.

3. 6N Ammonium hydroxide

Procedure (50)

The dried paper chromatogram strip was passed rapidly through the $AgNO_3$-acetone reagent solution twice, drying after each run through. The dry paper was then passed once through the ethanol-NaOH solution. Brown silver oxide was immediately produced. Reducing sugars formed dense black spots of silver at room temperature. When reduction was judged complete, excess silver oxide was dissolved by immersion of the strip in 6N ammonium hydroxide for a few minutes, after which it was washed with water and dried. The spots could have been rendered jet black by momentary exposure to H_2S.

The identity of the spots was determined by comparing the positions of the unknown spots with those of the standards. This method was more accurate than calculating the R_F values. Since some of the chromatograms were run for 48 hours and the solvent front had run off the paper, it was considered unreliable to calculate the ratio of the movement of a spot to the movement of the solvent front.

RESULTS

The results are presented in two sections. First, those obtained from paper chromatography. Second, those obtained from gas chromatography. The qualitative results obtained from paper chromatography are shown in Table II. Figures 3, 4, and 5 are representations of the actual paper chromatograms. The tentative qualitative results for gas chromatography are shown in Table III. Figures 6-14 are representations of the actual gas chromatograms. Because the results obtained from paper chromatography were sufficient for qualitative determinations of the monosaccharides in the extracellular polysaccharide hydrolysates, not much emphasis was placed on the gas chromatography. Only preliminary gas chromatography experiments were carried out. The identities of the peaks were determined tentatively by first, assuming that the peaks represented the monosaccharides that were shown to be present by the paper chromatographic examinations of the hydrolysates. Secondly, the individual peak retention times were compared with the retention times of known monosaccharides chromatographed under similar conditions (17, 37, 49). However, more positive identification could have been obtained by experiments using the

peak enhancement technique. This involves adding a known monosaccharide to the hydrolysate and then observing which peaks are enhanced on the gas chromatograms. The peaks enhanced would most likely be those of the known monosaccharide that was added. This process would be carried out for all the monosaccharides thought to be present in the hydrolysate. The gas and paper chromatography results appear to be the same. But, until this systematic kind of identification involving peak enhancement is carried out the gas chromatography results are inconclusive. However, the gas chromatograms should remain in the thesis. Researchers doing quantitative gas chromatography on the extracellular polysaccharides produced by some yeasts and yeast-like fungi may want to refer to and interpret further some of the gas chromatograms. (See Appendix page 62.)

Table II

PAPER CHROMATOGRAPHY RESULTS

Group	Fungus	Monosaccharides in the Extracellular Polysaccharide Hydrolysis Products			
		Galactose	Glucose	Mannose	Xylose
Group I Sub-group I	Cryptococcus laurentii NRRL YB-4920 (UBC #8114)	+	+	+	+
	Tremella mesenterica RJB #2259-6	+	+	+	+
	Bullera alba (Hanna) Derx UBC #983	+	+	+	+
	Sporobolomyces odorus UBC #949	+	+	+	+
	Sporobolomyces odorus UBC #981	+	+	+	+
	Rhodotorula glutinis UBC #940	+	+	+	+
	Sporobolomyces singularis UBC #8018	+	+	+	+
Group I Sub group II	Ustilago hordei UBC #570 (C.Person E3(-))	+	+	+	-
Group II	Taphrina populina UBC #249	-	+	+	-

Figure 3

Tremella mesenterica and Bullera alba. Separation of products using descending paper chromatography and solvents ethyl acetate: pyridine: water, (8:2:2).

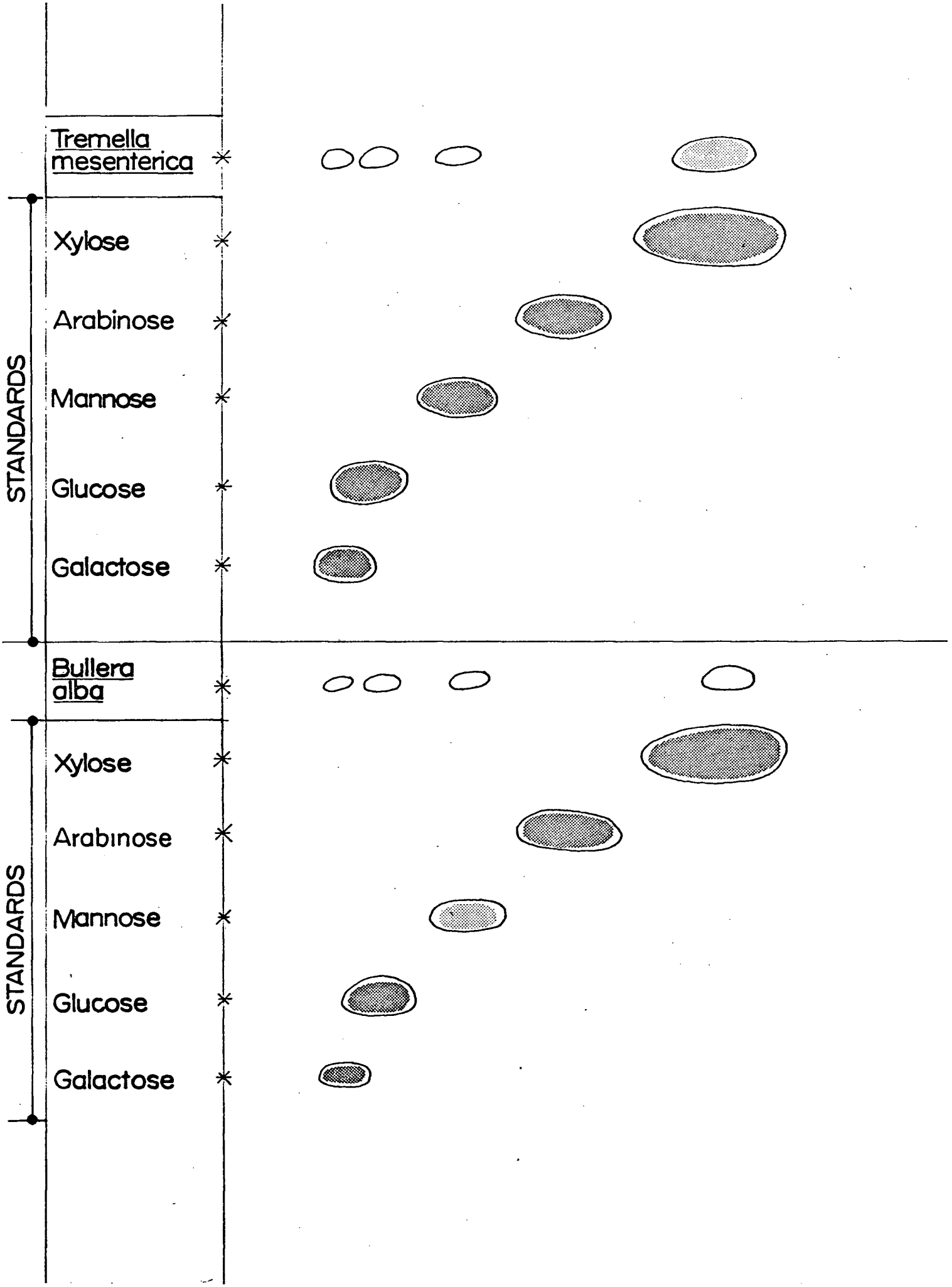

Tremella mesenterica
Xylose
Arabinose
Mannose
Glucose
Galactose
STANDARDS
Bullera alba
Xylose
Arabinose
Mannose
Glucose
Galactose
STANDARDS

Figure 4

Sporobolomyces singularis, Sporobolomyces odorus (#949), Sporobolomyces odorus (#981) Cryptococcus laurentii. Separation of products using descending paper chromatography and solvents ethyl acetate: pyridine: water, (8:2:2).

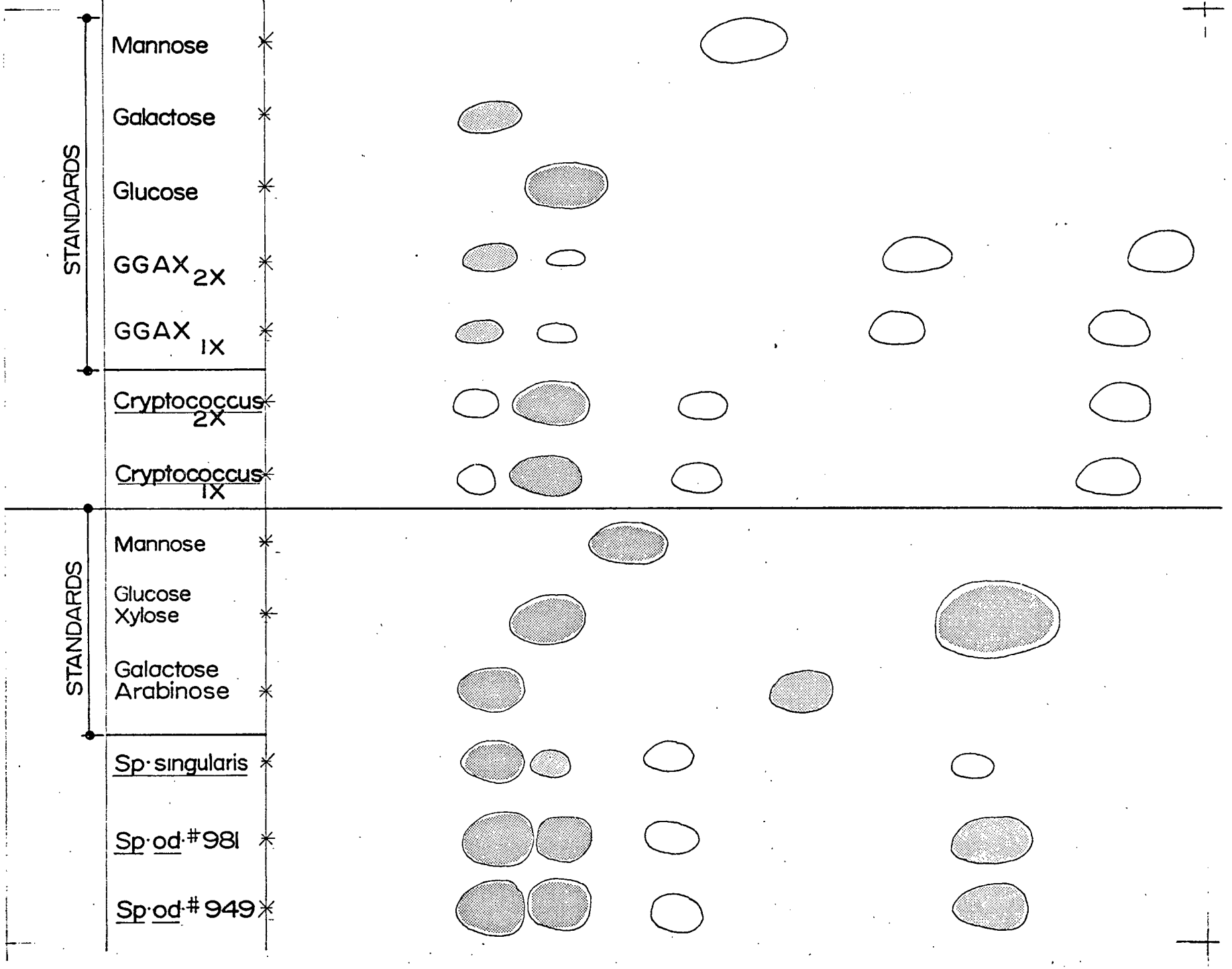

STANDARDS
Mannose
Galactose
Glucose
GGAX 2X
GGAX 1X
Cryptococcus 2X
Cryptococcus 1X
STANDARDS
Mannose
Glucose
Xylose
Galactose
Arabinose
Sp·singularis
Sp·od·#981
Sp·od·#949

Figure 5

Ustilago hordei, Rhodotorula glutinis, Sporobolomyces singularis, Taphrina populina. Separation of products using descending paper chromatography and solvents ethyl acetate: pyridine: water, (8:2:2).

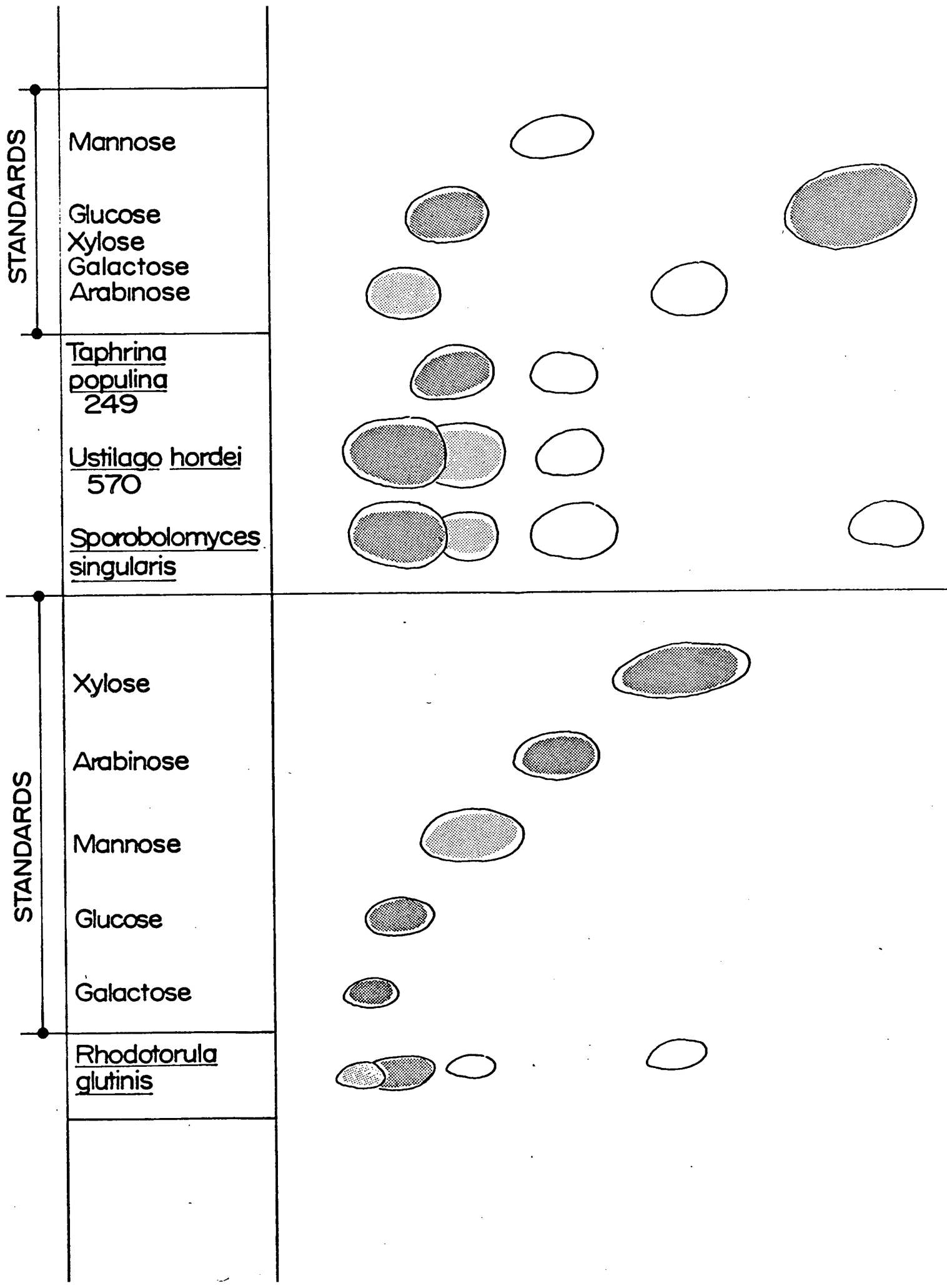

STANDARDS
Mannose
Glucose
Xylose
Galactose
Arabinose
Taphrina populina 249
Ustilago hordei 570
Sporobolomyces singularis
STANDARDS
Xylose
Arabinose
Mannose
Glucose
Galactose
Rhodotorula glutinis

Table III

Gas Chromatography Results

Fungus	Peak	Retention Time (Minutes)	Tentative Identification
Tremella mesenterica	1	2.9	Solvent
	2	13.8	Xylose
	3	17.5	Xylose
	4	19.8	Xylose
	5	22.6	Galactose, Mannose
	6	25.7	Galactose
	7	27.4	Galactose, Glucose, Mannose
	8	30.5	Glucose
Cryptococcus laurentii	1	19.7	Xylose
	2	23.0	Xylose
	3	26.6	Galactose, Mannose
	4	31.8	Galactose, Glucose, Mannose
	5	37.0	Glucose

Table III (Continued)

Gas Chromatography Results

Fungus	Peak	Retention Time (Minutes)	Tentative Identification
Bullera alba	1	2.9	solvent
	2	4.5	solvent
	3	6.2	solvent
	4	18.0	Xylose
	5	20.4	Xylose
	6	23.2	Galactose, Mannose.
	7	25.9	Galactose
	8	28.7	Galactose, Glucose, Mannose
	9	33.4	Glucose
Sporobolomyces odorus (#949)	1	17.4	Xylose
	2	19.1	Xylose
	3	21.2	Xylose
	4	24.0	Galactose, Mannose
	5	26.6	Galactose, Mannose
	6	29.3	Galactose
	7	31.9	Galactose, Glucose, Mannose
	8	37.0	Glucose

Table III (Continued)

Gas Chromatography Results

Fungus	Peak	Retention Time (Minutes)	Tentative Identification
Sporobolomyces odorus (#981)	1	17.8	Xylose
	2	19.6	Xylose
	3	22.0	Xylose
	4	28.1	Galactose, Mannose
	5	31.0	Galactose
	6	33.8	Galactose, Glucose, Mannose
	7	38.7	Glucose
Sporobolomyces singularis	1	18.8	Xylose
	2	28.2	Galactose, Mannose
	3	30.2	Galactose, Glucose, Mannose
	4	34.2	Glucose
Rhodotorula glutinis	1	3.0	Solvent
	2	15.3	Xylose
	3	17.3	Xylose
	4	22.2	Galactose, Mannose
	5	24.8	Galactose
	6	26.4	Galactose, Glucose, Mannose
	7	30.4	Glucose

Table III (Continued)

Gas Chromatography Results

Fungus	Peak	Retention Time (Minutes)	Tentative Identification
Ustilago hordei	1	23.5	Galactose, Mannose
	2	25.0	Galactose
	3	30.2	Galactose, Glucose, Mannose
	4	36.2	Glucose
Taphrina populina	1	14.7	Mannose
	2	23.5	Mannose
	3	24.3	Glucose
	4	29.0	Glucose
	5	50.3	unidentified

Figure 6

Tremella mesenterica. Separation of products as trimethylsilyl derivatives.

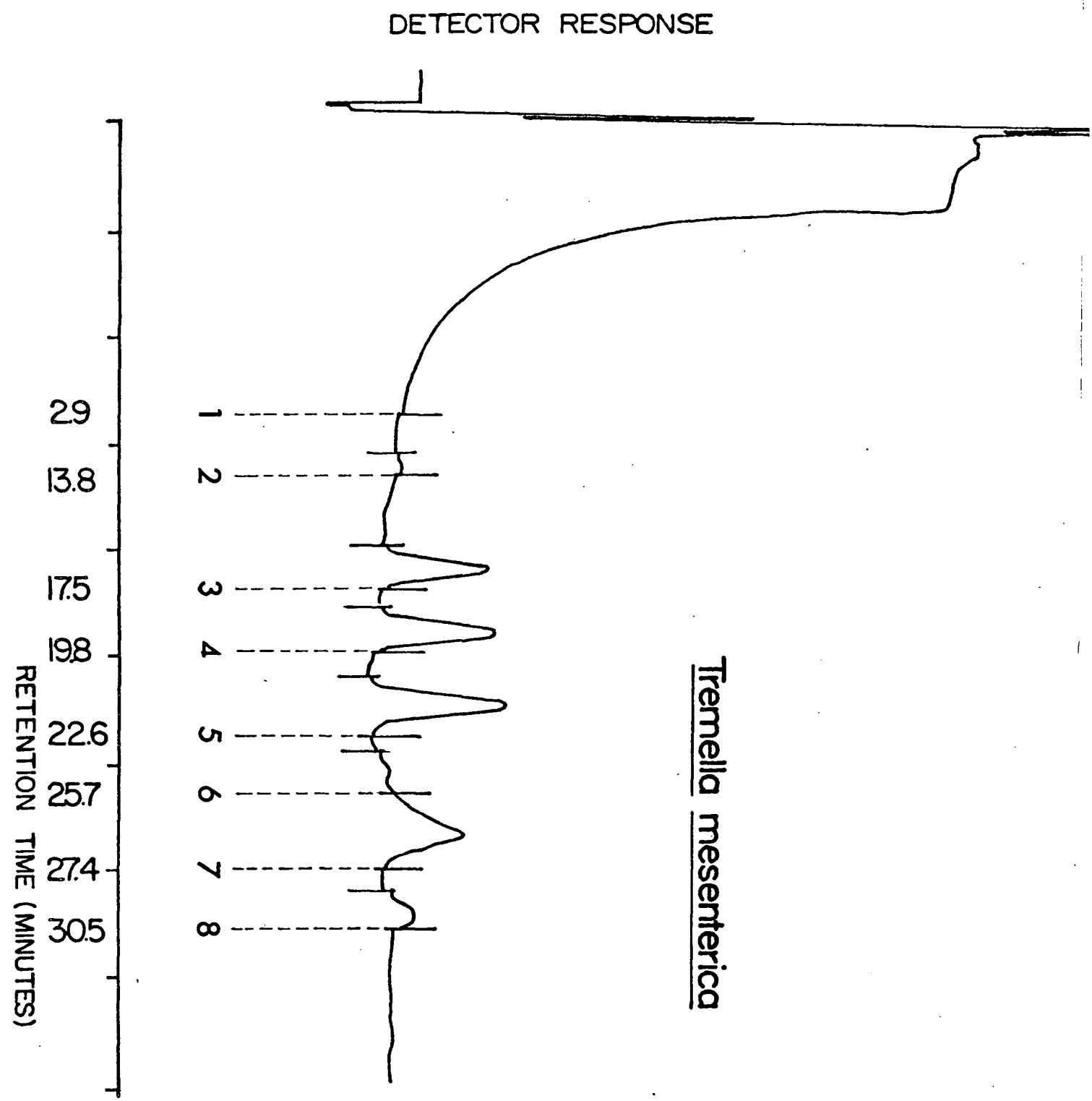

DETECTOR RESPONSE
RETENTION TIME (MINUTES)
2.9
13.8
17.5
19.8
22.6
25.7
27.4
30.5
1
2
3
4
5
6
7
8
Tremella mesenterica

Figure 7

Cryptococcus laurentii. Separation of products as trimethylsilyl derivatives.

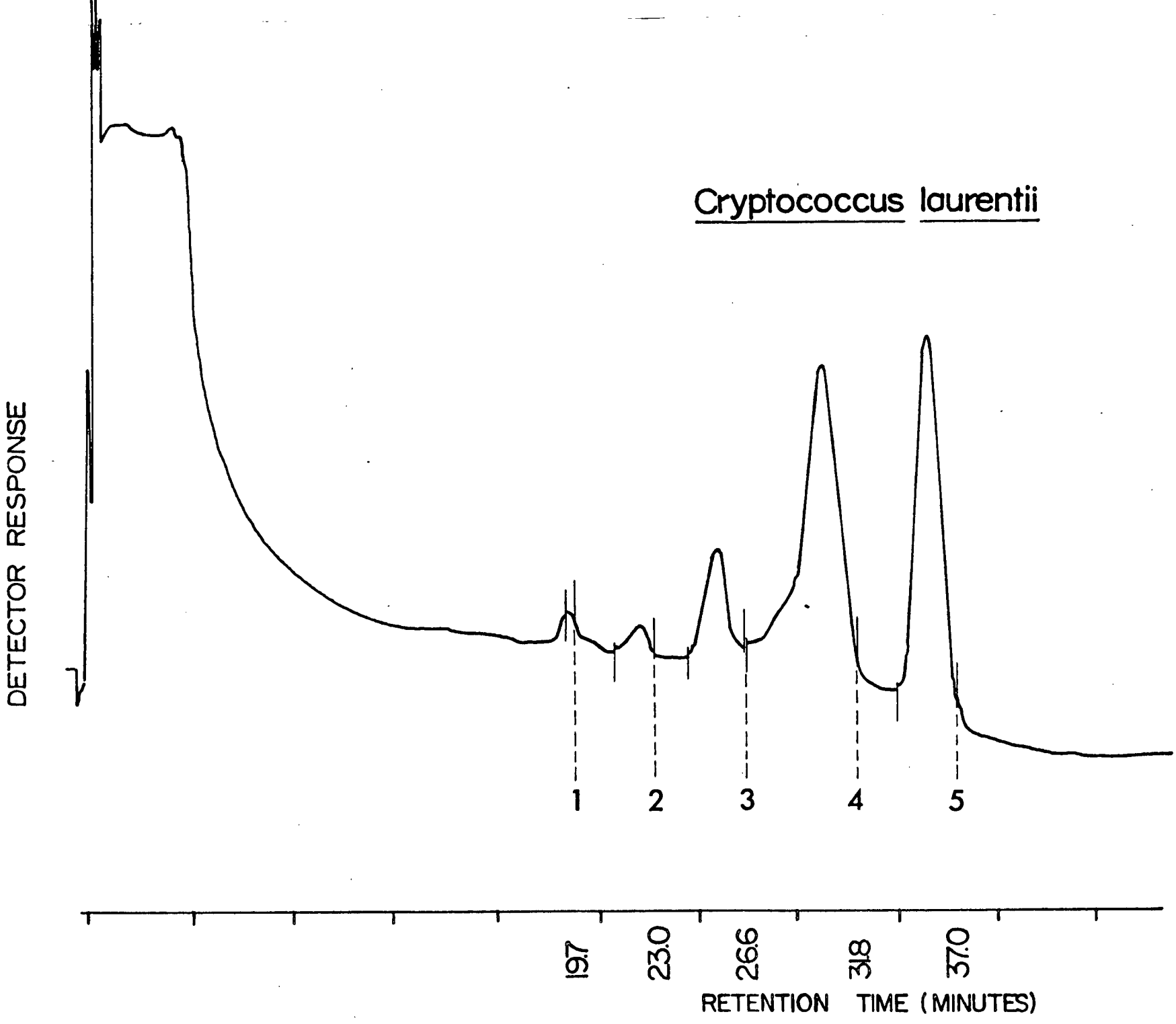

Cryptococcus laurentii
DETECTOR RESPONSE
1
2
3
4
5
19.7
23.0
26.6
31.8
37.0
RETENTION TIME (MINUTES)

Figure 8

Bullera alba. Separation of products as trimethylsilyl derivatives.

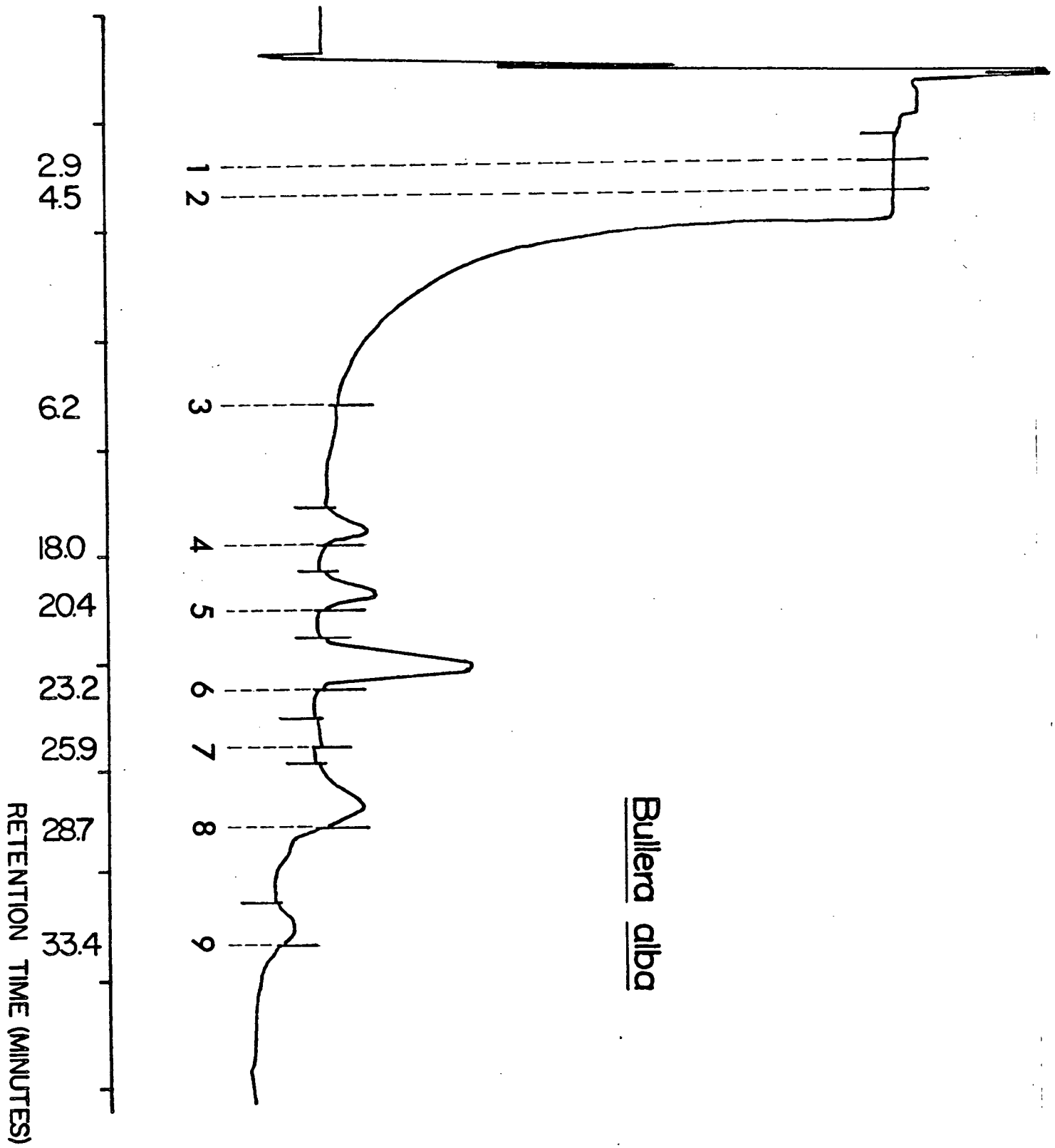

DETECTOR RESPONSE
2.9
4.5
6.2
18.0
20.4
23.2
25.9
28.7
33.4
RETENTION TIME (MINUTES)
1
2
3
4
5
6
7
8
9
Bullera alba

Figure 9

Sporobolomyces odorus (#949). Separation of products as trimethylsilyl derivatives.

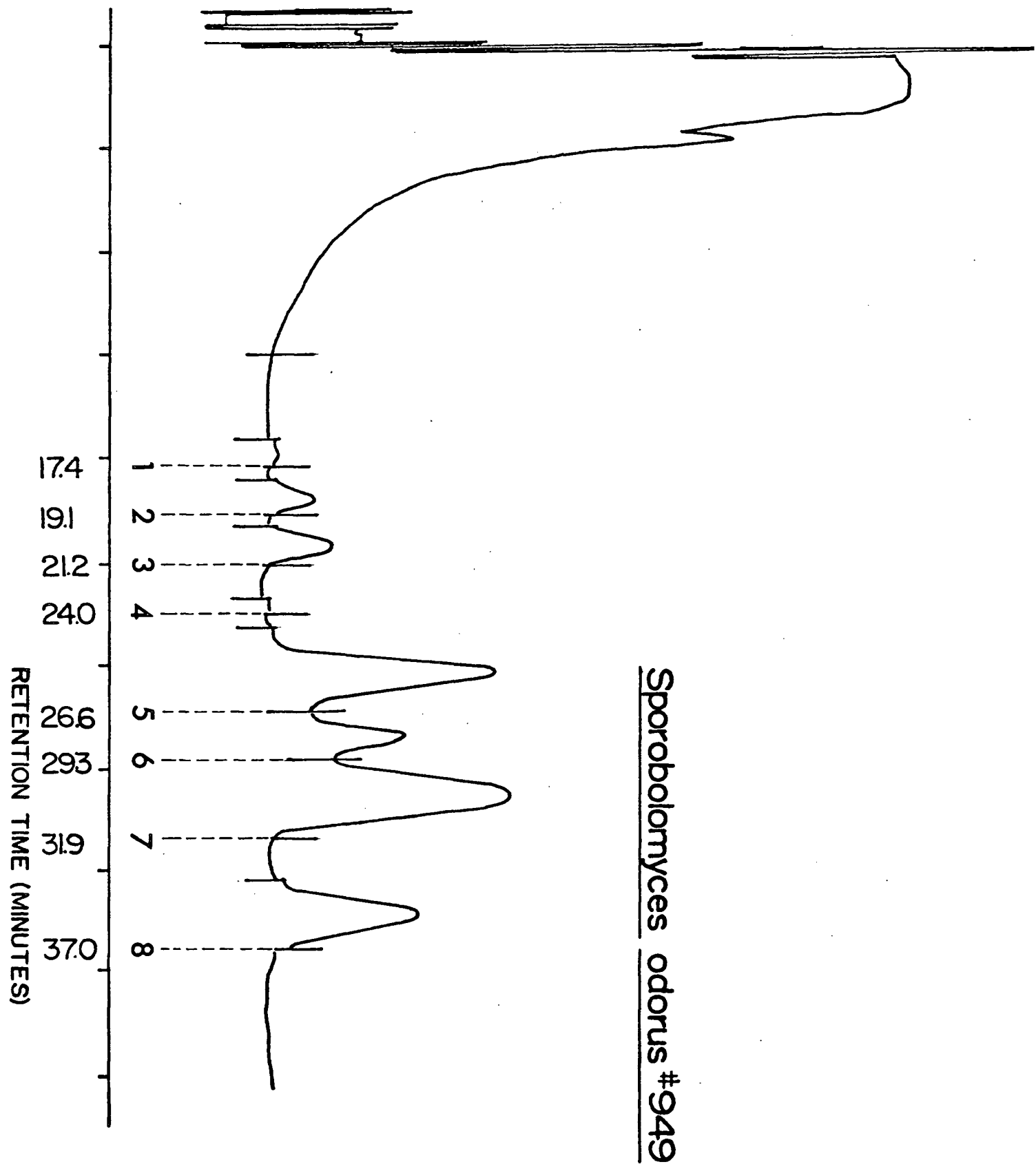

DETECTOR RESPONSE
17.4
19.1
21.2
24.0
26.6
29.3
31.9
37.0
1
2
3
4
5
6
7
8
RETENTION TIME (MINUTES)
Sporobolomyces odorus #949

Figure 10

Sporobolomyces odorus (#981). Separation of products as trimethylsilyl derivatives.

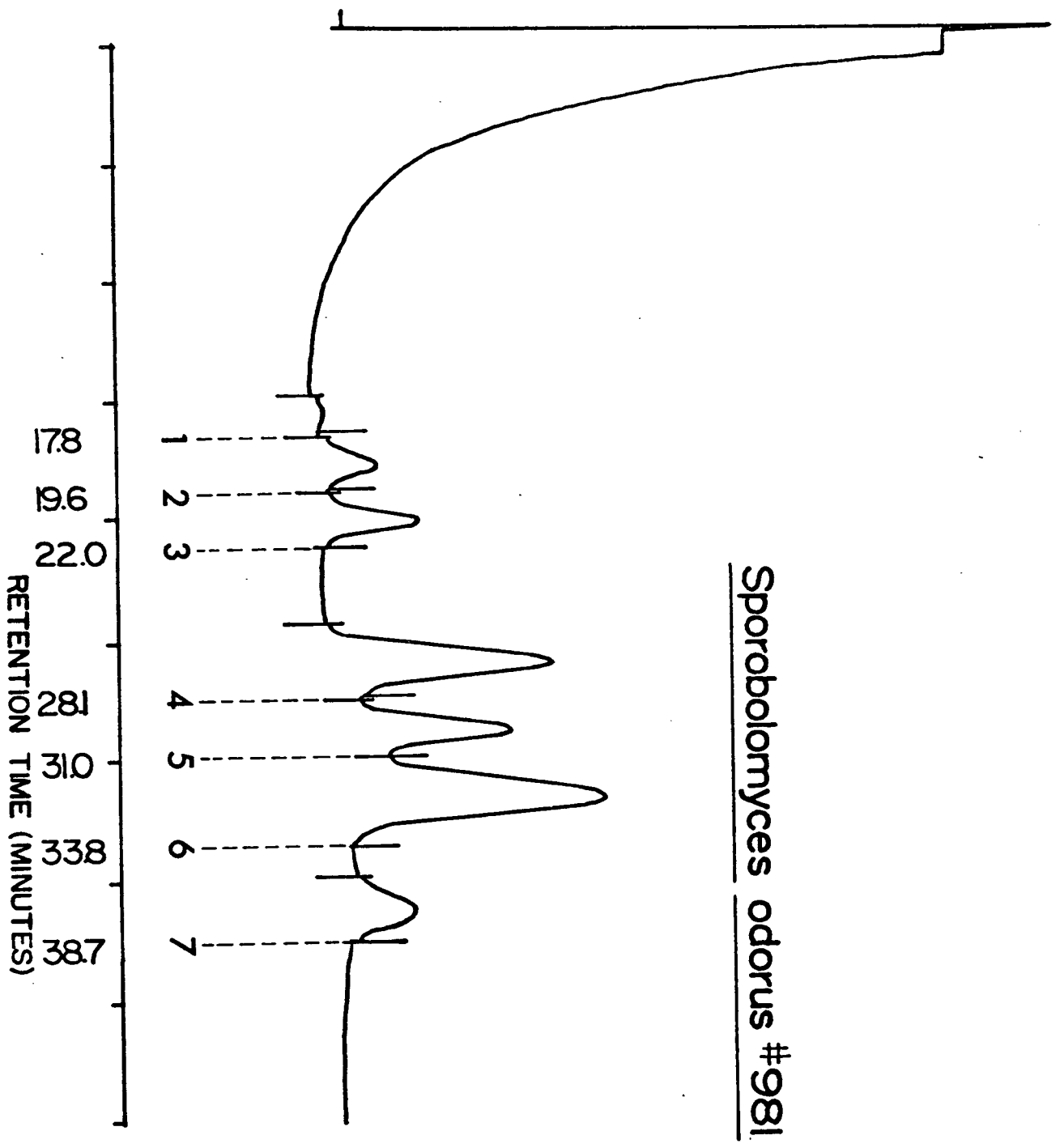

DETECTOR RESPONSE
RETENTION TIME (MINUTES)
17.8
19.6
22.0
28.1
31.0
33.8
38.7
1
2
3
4
5
6
7
Sporobolomyces odorus #981

Figure 11

Sporobolomyces singularis. Separation of products as trimethylsilyl derivatives.

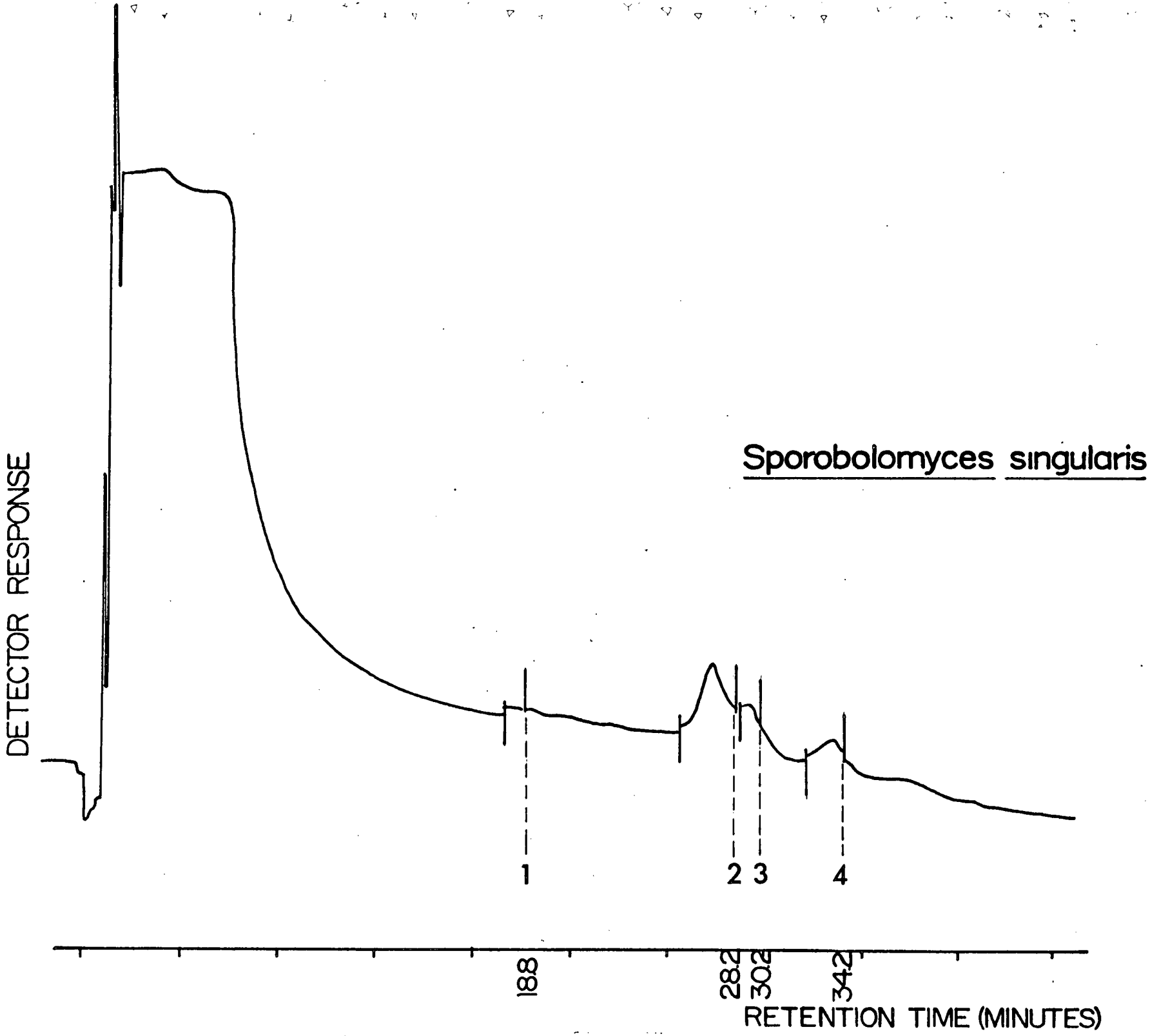

Sporobolomyces singularis
DETECTOR RESPONSE
1
2
3
4
18.8
28.2
30.2
34.2
RETENTION TIME (MINUTES)

Figure 12

Rhodotorula glutinis. Separation of products as trimethylsilyl derivatives.

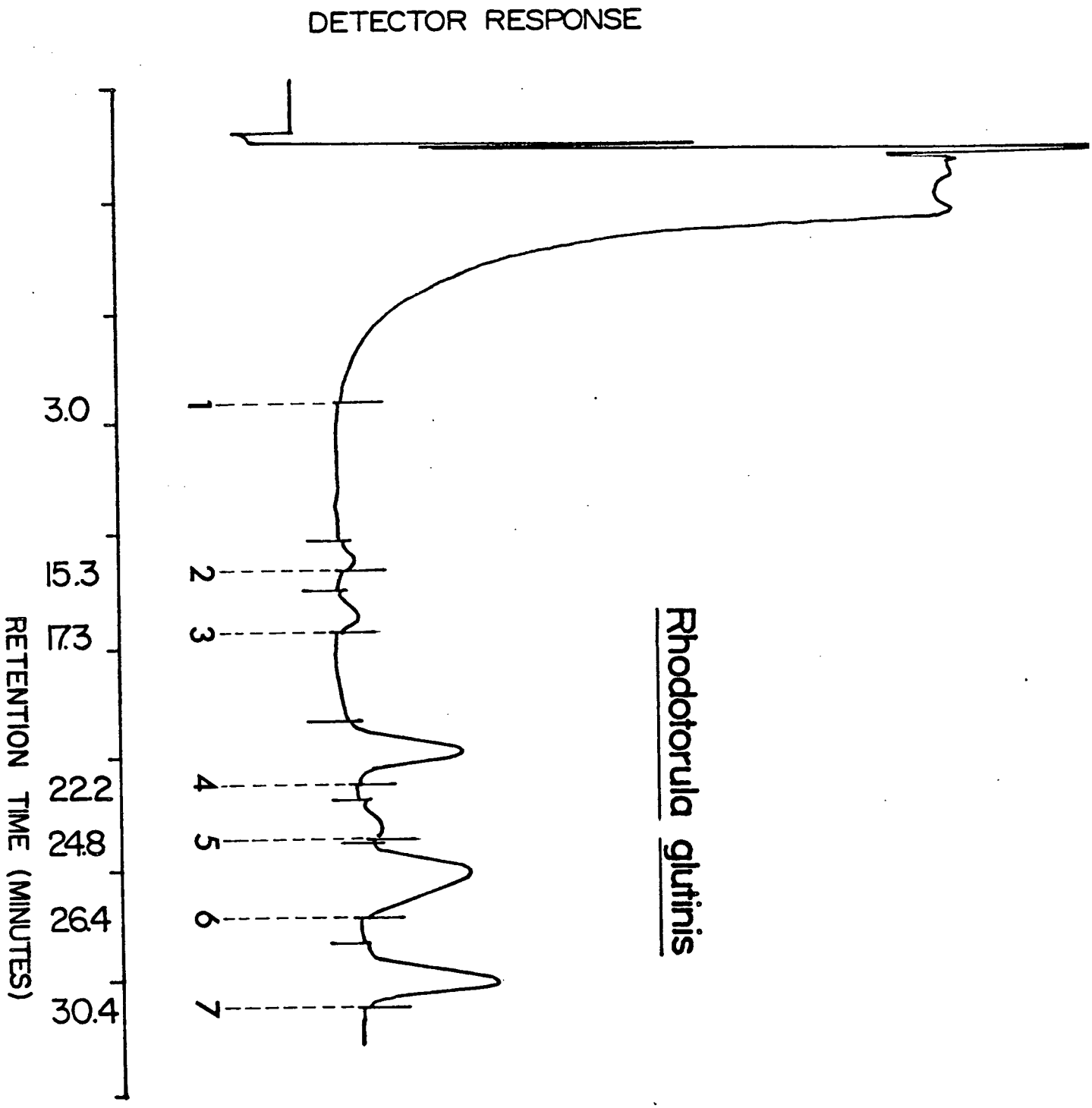

DETECTOR RESPONSE
RETENTION TIME (MINUTES)
3.0
15.3
17.3
22.2
24.8
26.4
30.4
1
2
3
4
5
6
7
Rhodotorula glutinis

Figure 13

Ustilago hordei. Separation of products as trimethylsilyl derivatives.

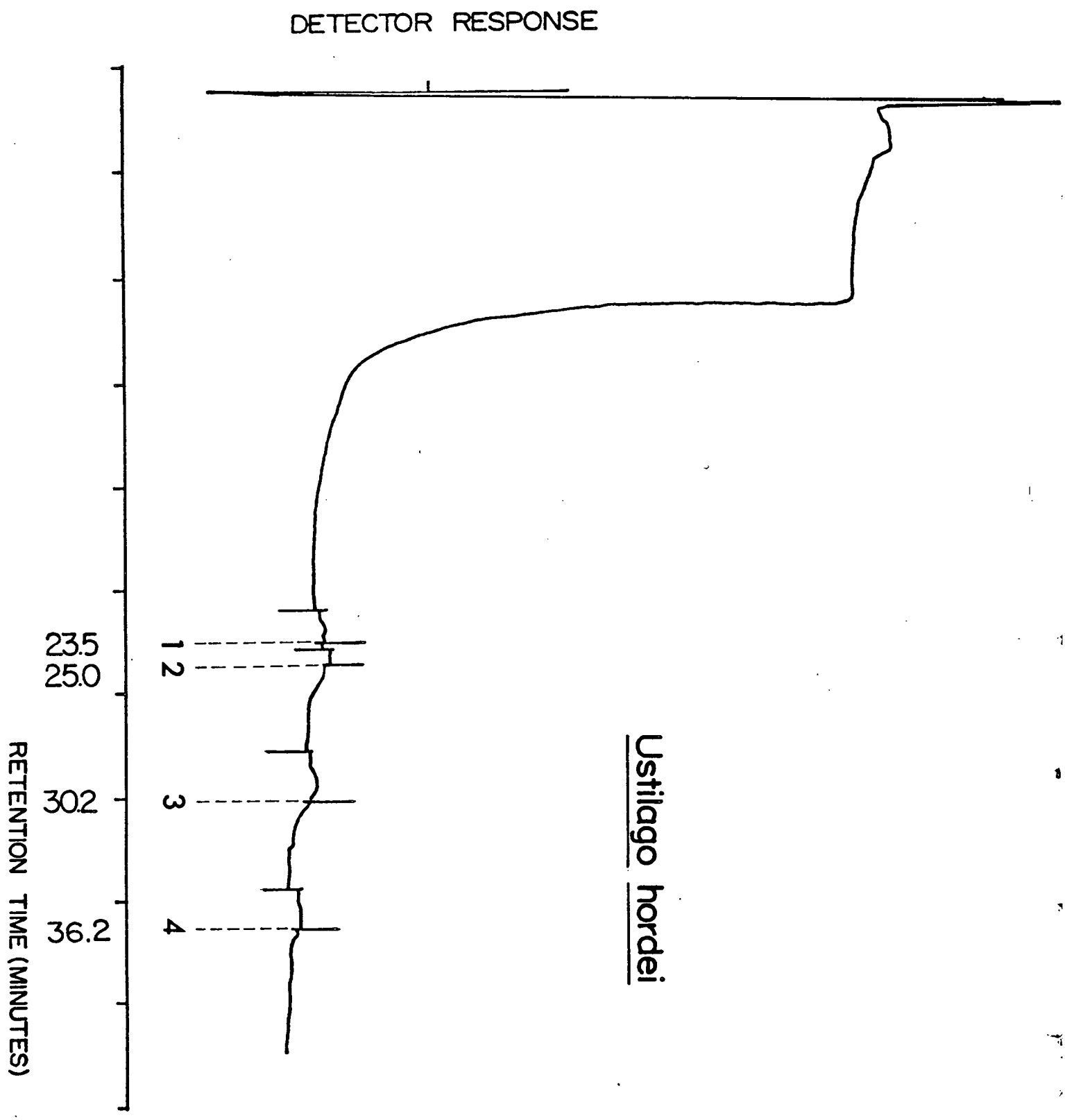
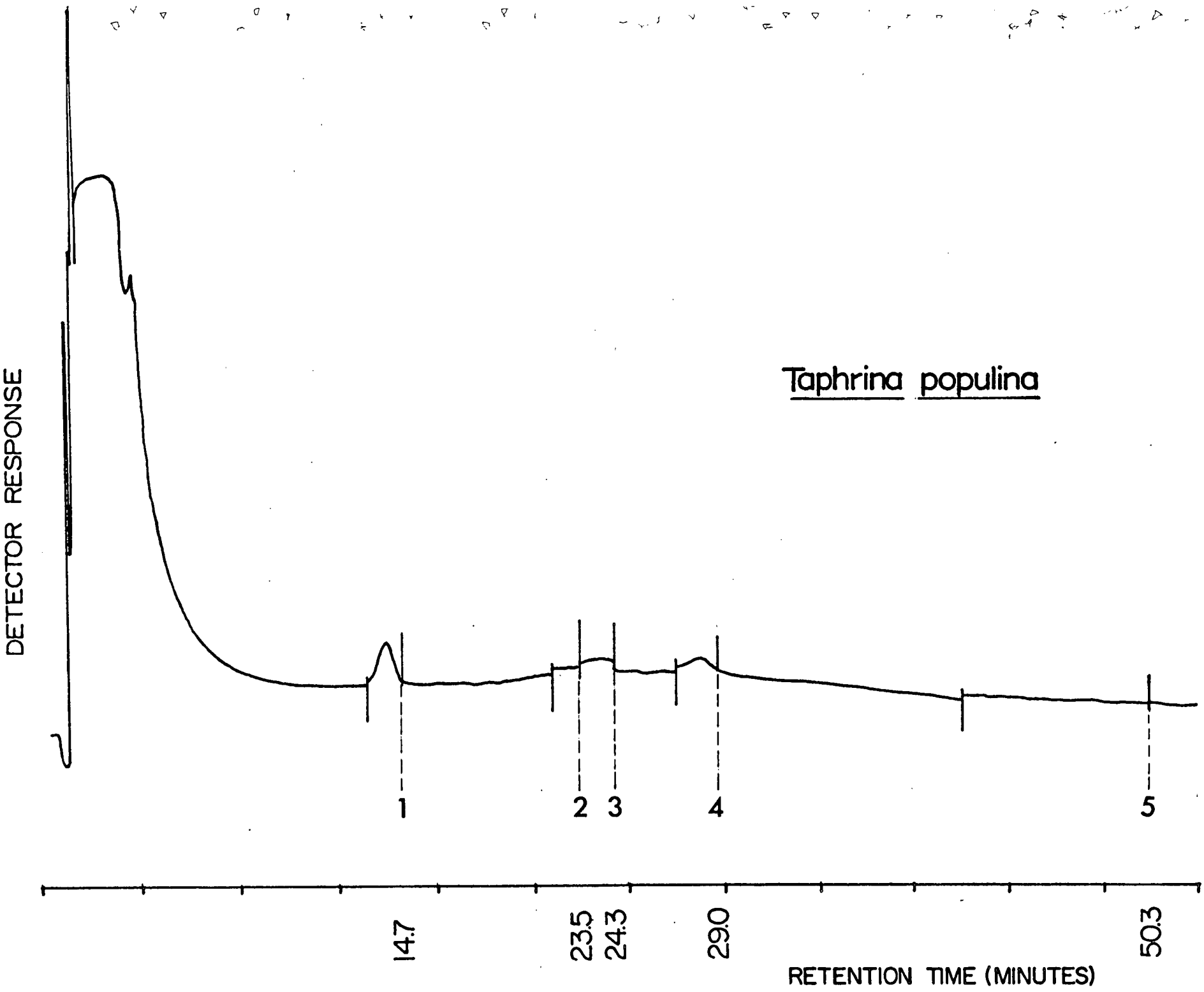

DETECTOR RESPONSE
RETENTION TIME (MINUTES)
23.5
25.0
30.2
36.2
1
2
3
4
Ustilago hordei

Figure 14

Taphrina populina. Separation of products as trimethylsilyl derivatives.


Taphrina populina
DETECTOR RESPONSE
1
2
3
4
5
14.7
23.5
24.3
29.0
50.3
RETENTION TIME (MINUTES)

DISCUSSION

The discussion relates my results to those of previous workers. It also makes some logical deductions. For convenience, the yeasts and yeast-like fungi investigated were assigned to two groups. Group I was divided into two subgroups. Fungi in Subgroup I were: Cryptococcus laurentii Tremella mesenterica, Bullera alba, Sporobolomyces odorus, Sporobolomyces singularis and Rhodotorula glutinis. The monosaccharides galactose, glucose, mannose and xylose were present in the hydrolysates of the extracellular polysaccharides produced by the fungi in Subgroup I. Subgroup II included Ustilago hordei with galactose, glucose and mannose present; xylose absent. Group II consisted of Taphrina populina with glucose and mannose present; galactose and xylose absent. Interestingly, the two groups formed above contain some fungi that have already been suggested as taxonomically related. A Cryptococcus-Tremella taxonomic relationship was first put forward by Kobayashi and Tubaki (28). Members of the two genera are similar in extracellular polysaccharide production, isolation and analysis (1, 19, 24, 44). They also have similar carbon assimilation patterns and produce

starch (32, 41). Similar morphology and enzymatic xylosylation reactions are also important (3, 13, 28, 32, 41).

My results present biochemical information that may support the relationship already suggested between Cryptococcus laurentii and Tremella mesenterica (41). I found the monosaccharides galactose, glucose, mannose and xylose to be present in the hydrolysates of the extracellular polysaccharides produced by both Cryptococcus laurentii and Tremella mesenterica. The presence of glucose, mannose, and xylose is in agreement with the results of previous workers who found both an acidic xylomannan and a neutral glucan to be present in the extracellular polysaccharide hydrolysate of both Cryptococcus laurentii and Tremella mesenterica (1, 19, 24, 41, 44). Galactose had been reported only in trace amounts from Cryptococcus laurentii (24) and had not been reported previously from Tremella mesenterica. Galactose may have been present as a contaminant from cell wall or endocellular material from yeast cells that might have broken down during culturing or centrifugation. Or, possibly, galactose is more tightly bound to the cell wall than the other monosaccharides and under certain conditions has not been previously isolated and analyzed. Perhaps an electron microscopist working on fungal cell walls and using

radioactive tracers would be able to resolve this. Though Tremella mesenterica has not yet been shown to contain 1-3 linkages in its extracellular polysaccharide (19), as has Cryptococcus laurentii there is still substantial evidence to support a relationship between these two species.

Members of the genera Bullera and Cryptococcus have been suggested as being taxonomically related on the bases of similar general morphology and lack of pseudomycelium; starch production and carbon assimilation. Both genera assimilate inositol and break the α-glucosidic bonds of melibiose, melezitose, and methylα-D-glucoside. They also both produce starch and lack pseudomycelium. If Bullera cultures had not been known to produce ballistospores, some of them might have been included in the genus Cryptococcus (32). My findings that the monosaccharides of Bullera alba are qualitatively the same as those of Cryptococcus laurentii may give added support to the Bullera-Cryptococcus relationships in general. However, the results pertain more directly to giving biochemical support to a Bullera alba and Cryptococcus laurentii relationship.

As shown in my results, both Sporobolomyces singularis and Sporobolomyces odorus produced an extracellular polysaccharide with the monosaccharides galactose, glucose, mannose

and xylose in the hydrolysate. The presence of galactose and glucose in the Sporobolomyces singularis extracellular polysaccharide hydrolysate is in agreement with the results of Gorin et al (21). The finding of mannose in the extracellular polysaccharide hydrolysate of Sporobolomyces singularis is in agreement with the results of Phaff and Spencer (36). Xylose had not been reported previously to be present in the Sporobolomyces singularis hydrolysate. The extracellular polysaccharide produced by Sporobolomyces odorus had not been previously analyzed.

The monosaccharides that I found to be present in the hydrolysate of the extracellular polysaccharide produced by Rhodotorula glutinis were galactose, glucose, mannose and xylose. Mannose may be part of a mannan similar to those mannans produced by both Rhodotorula glutinis and Sporobolomyces singularis (20, 36). Gorin et al stated that in addition to the mannan, Rhodotorula glutinis produced a hexose and a methyl pentose. Perhaps either galactose or glucose that I determined to be present in the Rhodotorula glutinis extracellular polysaccharide hydrolysate could be the same as the unidentified hexose of Gorin et al (20). Also, xylose, the pentose that I found to be present in the Rhodotorula glutinis extracellular hydrolysate could be a

derivative of the unidentified methyl pentose of Gorin et al (20).

The qualitative results for the Ustilago hordei extracellular polysaccharide are new. The monosaccharides present were: galactose, glucose and mannose. Xylose was absent.

Indications were that quantitative, instead of qualitative analyses using gas chromatography might be more important in determining what species of Sporobolomyces and Rhodotorula are most closely related. A duality amongst some members of both Rhodotorula and Sporobolomyces that was suggested by antigenic and percent G+C analyses (32, 33, 48, 51), may be supported by quantitative gas chromatographic analyses.

The results for Taphrina populina are new. In this case the qualitative analyses may prove to be another useful means of distinguishing Taphrina species from those of Rhodotorula and Cryptococcus. However, more analyses of other species are necessary to determine whether this is so. My results can only distinguish Taphrina populina from Rhodotorula glutinis and Cryptococcus laurentii. Some of the known tests for distinguishing these genera at the moment are carbon assimilation and observation of starch

production. Taphrina is inositol negative and produces starch; Rhodotorula is starch negative; and Cryptococcus is inositol positive (32).

Taphrina and Lipomyces species have been postulated as taxonomically related because of similarities in starch production, and multispores asci found in both genera (55). However, the development of the asci in Taphrina was shown by Kramer (29) to be altogether different from those in Lipomyces. My results showed that Taphrina populina produced an extracellular polysaccharide with the monosaccharides glucose and mannose present in the hydrolysis product. But Slodki and Wickerham showed that the Lipomyces extracellular polysaccharide was composed of mannose only (42). The Lipomyces-Taphrina relationship seems doubtful at this point.

Barnett, in 1957 (5), stated that there were four major unsolved problems of yeast taxonomy. These were that: (1) There is a need to devise different sets of biochemical tests for different groups of yeasts. (2) It would be helpful to have a quantitative evaluation of how many tests are necessary to separate different strains into already named groups, say species. (3) The biochemical activity described should be considered quantitatively.

(4) The conditions for each of these tests needs to be assessed critically and labile characters should be avoided. In 1961, Barnett (6) suggested that a classification should be developed in which there are no sporulation tests, morphological criteria are given greater precision, and biochemical tests are designed to give more information. He stated that the Dutch classification has three major weaknesses. These are: (1) Sporulation tests are given supreme importance, arrays of media are used, and a search for spores is necessary. Negative tests are equivocable. (2) Morphological criteria are ill defined and difficult to specify precisely. (3) Biochemical tests are crude. In 1966, Barnett (7) stated that: (1) the presence of enzymes in some yeasts and absence in others can lead to correlations between the results of many other tests. (2) analyses of the results of large numbers of tests could be used to make evident the biochemical features that are likely to underlie those correlations.

With reference to my experiments, it is evident that a quantitative analysis using GLC would have been better for distinguishing between species of the same genus. e.g. Sporobolomyces singularis and Sporobolomyces odorus. It is also apparent that to be statistically correct in implying

taxonomic relationships, researchers should use the same methods and techniques for culturing the fungi and for isolating and analyzing the extracellular polysaccharide from those fungi that are to be compared. Variations in the properties of the polysaccharide may be dependent on the fermentative conditions. These variations are related to the constitution and molecular size of the polymer (24). A short discussion of fermentative conditions that are of importance in this respect is relevant here. First, consider the growth period. Acidic xylomannan was produced by Tremella mesenterica at seven days (19), and by Cryptococcus laurentii at four (44), five (24) and six days (12). Neutral glucan, was produced only after fourteen days growth by both Cryptococcus laurentii and Tremella mesenterica (1, 19). Sporobolomyces species produced phosphorylated galactans at twelve days (43). Secondly, the pH of the medium is an important factor to consider. An initial pH of 6.4 to 6.8 was suitable for polymer production by Cryptococcus laurentii (1, 12) and a pH of 6.0 was required for phosphorylated galactan production by Sporobolomyces species (43). Other workers found that Sporobolomyces singularis had good growth at pH 6.0 but no polysaccharide production. Yields of trisaccharide and tetrasaccharide increased at pH 3.75.

Above pH 4.0 no oligosaccharide was formed (21). Temperature is a third condition of importance in extracellular polysaccharide production. Maximum polymer production by Cryptococcus laurentii occurred at 25°C (12); by Sporobolomyces singularis at 22°C (21); and by Rhodotorula glutinis at 20°C (12); by Sporobolomyces singularis at 22°C (21); and by Rhodotorula glutinis at 20°C and 24°C (20). Fourthly, aeration and other factors such as traces of $MnSO_4$ increased the polysaccharide production by Cryptococcus laurentii (12). In most cases the medium, temperature, pH, aeration, and growth period were kept constant so that analytical results might be statistically comparable. Because the structure and contents of the extracellular polysaccharides are so closely governed by the cultural conditions it is apparent that the analyses of the extracellular polysaccharides may not be one of the best parameters to use taxonomically. Perhaps a study encompassing quite a few biochemical parameters would be valuable. This is because the researcher would be able to select those parameters that were least variable and use them taxonomically. For instance, the qualitative or quantitative analyses of the cell wall amino acids might be more useful than the analyses of the extracellular polysaccharides.

With regard to the Cryptococcus-Tremella relationships: should the fact that Tremella mesenterica has not been shown to have 1-3 linkages in its extracellular polysaccharide as has Cryptococcus laurentii (19) be weighted heavily against the proposed taxonomic relationship? In other words, how many tests are necessary to prove the relationship between Cryptococcus laurentii and Tremella mesenterica? Personally I don't think that the fact that Tremella mesenterica has not been shown to have 1-3 linkages in its extracellular polysaccharide as has Cryptococcus laurentii should be weighted heavily against this relationship. Johnson made a statement that I think is quite fitting. He said that no optimal classification can be defined, but improvement is possible up to the point of inherent instability (25).

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APPENDIX

The appendix presents quantitative results that were not included in the main part of the thesis. First, quantitative gas chromatography results. The fact that the identities of the peaks in the gas chromatograms are only tentative should be taken into consideration. Table IV shows the areas of the peaks on the gas chromatograms. Table V shows the percent monosaccharide of the total sugar. These percentages were calculated using correction factors for the monosaccharides in equilibrium solutions (17,37,49). Second, some mention of quantitative yields of crude extracellular polysaccharides should be made. *Tremella mesenterica*, *Cryptococcus laurentii*, *Bullera alba*, *Sporobolomyces odorus*, *Sporobolomyces singularis*, and *Rhodotorula glutinis* all produced approximately one to two grams of crude polysaccharide in approximately six litres of culture medium. *Taphrina populina* and *Ustilago hordei* both produced less than one gram of crude polysaccharide in six litres of culture medium.

Table IV

Fungus	Peak Number	Tentative Identification	Peak Area
Tremella mesenterica	1	Solvent	---
	2	Xylose	235
	3	Xylose	7,040
	4	Xylose	7,847
	5	Galactose, Mannose	11,610
	6	Galactose	1,241
	7	Galactose, Glucose, Mannose	10,594
	8	Glucose	1,414
Total Area			39,981
Cryptococcus laurentii	1	Xylose	376
	2	Xylose	7,943
	3	Galactose, Mannose	40,763
	4	Galactose, Glucose, Mannose	251,730
	5	Glucose	266,866
Total Area			567,678

Table IV (continued)

Fungus	Peak Number	Tentative Identification	Peak Area
Bullera alba	1	Solvent	
	2	Solvent	
	3	Solvent	
	4	Xylose	2,701
	5	Xylose	3,548
	6	Galactose, Mannose	13,568
	7	Galactose	495
	8	Galactose, Glucose, Mannose	5,564
	9	Glucose	1,412
Total Area			27,288
Sporobolomyces odorus (#949)	1	Xylose	389
	2	Xylose	2,439
	3	Xylose	3,953
	4	Galactose, Mannose	71
	5	Galactose, Mannose	22,724
	6	Galactose	12,306
	7	Galactose, Glucose, Mannose	36,460
	8	Glucose	15,072
Total Area			93,414

Table IV (continued)

Fungus	Peak Number	Tentative Identification	Peak Area
Sporobolomyces odorus (#981)	1	Xylose	1,007
	2	Xylose	4,691
	3	Xylose	8,074
	4	Galactose, Mannose	27,010
	5	Galactose	19,782
	6	Galactose, Glucose, Mannose	50,024
	7	Glucose	6,766
Total Area			117,354
Sporobolomyces singularis	1	Xylose	287
	2	Galactose, Mannose	6,357
	3	Galactose, Glucose, Mannose	1,095
	4	Glucose	1,471
Total Area			9,210
Rhodotorula glutinis	1	Solvent	---
	2	Xylose	688
	3	Xylose	1,378
	4	Galactose, Mannose	9,724
	5	Galactose	1,537
	6	Galactose, Glucose, Mannose	14,368
	7	Glucose	11,709
Total Area			39,404

Table IV (continued)

Fungus	Peak Number	Tentative Identification	Peak Area
Ustilago hordei	1	Galactose, Mannose	527
	2	Galactose	190
	3	Galactose, Glucose, Mannose	1,233
	4	Glucose	653
Total Area			2,603
Taphrina populina	1	Mannose	3,469
	2	Mannose	255
	3	Glucose	1,374
	4	Glucose	1,567
Total Area			6,665

Table V

Fungus	Monosaccharide	Percent of Total Sugar
Tremella mesenterica	Xylose	38.00
	Galactose	9.50
	Mannose	46.90
	Glucose	5.60
Cryptococcus laurentii	Xylose	1.40
	Galactose, Mannose	22.40
	Glucose	76.20
Bullera alba	Xylose	23.00
	Galactose	6.16
	Mannose	62.40
	Glucose	8.44
Sporobolomyces odorus (#949)	Xylose	7.24
	Galactose	45.10
	Mannose	21.36
	Glucose	26.30
Sporobolomyces odorus (#981)	Xylose	11.70
	Galactose	57.50
	Mannose	21.39
	Glucose	9.41

Fungus	Monosaccharide	Percent of Total Sugar
Sporobolomyces singularis	Xylose	3.30
	Galactose, Mannose	70.76
	Glucose	25.94
Rhodotorula glutinis	Xylose	5.20
	Galactose	13.34
	Mannose	33.16
	Glucose	48.30
Ustilago hordei	Galactose	25.00
	Mannose	34.30
	Glucose	40.70
Taphrina populina	Mannose	56.00
	Glucose	44.00