

THE PRODUCTION OF PENTOSE-CONTAINING POLY-SACCHARIDES BY *TORULOPSIS FLAVESCENS*

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Although the dextrans have become established as satisfactory "plasma extenders," they do have certain objectionable properties for this particular role. A group of polysaccharides which has not been explored as possible plasma extenders is that of the pentosans, especially those produced by microorganisms. It is conceivable that a pentosan could maintain the blood volume for longer periods of time than a dextran since pentosans should not be so readily hydrolyzed and metabolized by the body. It is also possible that pentosans may lack the antigenic properties of dextrans with certain structures (Kabat and Berg, 1953). In addition, a sugar polymer such as pentosan would represent a more physiological type of substance than compounds such as polyvinylpyrrolidone and presumedly would present less danger of potential toxicity than the latter polymer.

While the above presumptions would require rather extensive investigation, the present work was nevertheless initiated with the object of examining, in a preliminary fashion, the pentose-containing polysaccharides produced by a number of yeasts. Xylose polymers, especially plant xylan, is well-known and has been carefully characterized (Whistler, 1951). In addition, the polysaccharides of green algae (Lewin, 1956), fungi (Martin and Adams, 1956), and pathogenic yeasts (Evans and Theriault, 1953) have been shown to contain pentoses. Since Mager (1947) and Mager and Aschner (1947) reported that a number of non-pathogenic yeasts, especially of the species of *Torulopsis*, produce pentose-containing polysaccharides, various species of this genus were chosen for investigation.

METHODS AND MATERIALS

Strain of organism used.—A number of cultures (kindly provided by Dr. L. J. Wickerham of the Northern Utilization Research Branch, U.S.D.A., Peoria, Illinois) of various species of *Torulopsis* were surveyed for the ability to produce polysaccharides. Four of the cultures investigated produced a polysaccharide material which yielded, upon acid hydrolysis, a component moving similarly to xylose on paper chromatograms. Of these *T. flavescens* NRRL Y-1401 produced the highest yield of polysaccharide and was, therefore, selected for further study.

Culture media.—The original screening work was done using a complex medium with the following composition (Juni, 1951); glucose, 45 gm; Difco Proteose-peptone, 3 gm; Difco yeast extract, 3 gm; dibasic potassium phosphate, 4 gm; monobasic potassium phosphate, 4 gm; and distilled water, 1 lit. The pH after autoclaving was 7. The cultures were continuously aerated by incubating on a rotary shaker for 4 days at 30°C. In the later work, it was desired to use media which were synthetic or nearly so and for this purpose, the following were used: a) *Modified Wickerham's synthetic yeast medium (1946, 1949)*. This medium consisted of 20 ml of Difco Carbon Base (11.7 gm per 100 ml; autoclaved for 15 min); 15 ml of 46 percent glucose solution (sterilized by autoclaving); and 165 ml of a solution consisting of dibasic potassium phosphate, 0.3 gm; Bacto Casamino acids, 0.6 gm; potassium nitrate, 0.16 gm. This solution was also separately sterilized by autoclaving. b) *Glucose-Casamino acids-salts medium*. This medium represented a simplification of the above medium and consisted of

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Casamino acids, 3 gm; monobasic potassium phosphate, 4 gm; dibasic potassium phosphate, 3 gm; magnesium sulfate heptahydrate 0.5 gm; trace element solution, 5 ml; and distilled water, 1 lit. The trace element solution consisted of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 gm; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 gm; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.5 gm; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 gm and distilled water, 1 lit. After autoclaving of the above medium, 100 ml of 50 percent glucose, separately sterilized, were added. The pH of the medium (minus glucose) was adjusted to 7.0 before autoclaving.

Isolation of polysaccharides.—The method (after Mager, 1947) employed for recovering the polysaccharides from the culture media consisted of the following steps. a) The cultures were centrifuged in order to remove the yeast cells. b) The medium was concentrated on a steam bath and to this concentrate were added 3 volumes of 95 percent ethanol ($\frac{v}{v}$) and 10 gm of anhydrous sodium acetate per 100 ml of concentrate. The mixture was then placed in the cold over night at 5–10°C. c) The polysaccharide precipitate was recovered by centrifugation and triturated with 70% ethyl alcohol ($\frac{v}{v}$) containing 5 ml of glacial acetic acid per 100 ml. This mixture was centrifuged and the precipitate washed with absolute alcohol, then ether, and allowed to air dry. The product was an amorphous white to slightly tannish powder. This procedure was slightly modified for *T. flavescens* cultures since the latter were highly viscous, making concentration of the medium difficult. The concentration step was omitted, resulting only in the need for using more alcohol and sodium acetate because of the larger volume of culture supernatant. The remainder of the procedure was unmodified.

In order to ascertain the identities of the constituent monosaccharides of the materials isolated, paper chromatographic analyses were applied to the acid hydrolysates of the polysaccharides. Usually, about 50 mg of the polysaccharide were treated with 1 ml of 1.0N sulfuric acid and heated in a boiling water bath for 1 to 2 hr. The mixture was neutralized with barium carbonate and the supernatant obtained by centrifugation was applied to paper chromatograms. Descending development was employed using, as the mobile phase, the organic phase of a mixture of ethyl acetate, acetic acid and water in a ratio of 3:1:3, respectively. The monosaccharides were located on the paper by aid of the spray described by Horrocks and Manning (1949).

Preliminary attempts at fractionation.—As a preliminary attempt to estimate the homogeneity of the polysaccharide materials obtained, the following fractionation procedure was employed. About 250 mg of crude polysaccharide were suspended in 10 ml of 2N HCl, and heated in a boiling water bath until a stable, homogeneous suspension occurred (approximately 5 min). The suspension was cooled and centrifuged, resulting in a small amount of tan precipitate. The latter was designated Fraction A. The supernatant was neutralized to pH 6.5 with 50 percent KOH and a 0.67 volume of ethanol was added. A white precipitate resulted and was recovered. This precipitate was designated Fraction B. To the supernatant, two more volumes of alcohol were added and the precipitate, Fraction C was recovered. All three precipitates were washed respectively in 70 percent alcohol (containing 5 ml of glacial acetic acid per 100 ml), absolute alcohol, ether, and were then air dried.

RESULTS

Conditions for growth and polysaccharide production.—Although the highly complex glucose-proteose-peptone-yeast extract medium was very satisfactory for both growth and polysaccharide production, I felt that a synthetic medium should be devised so that separation of the polysaccharide from other organic materials would be facilitated. In addition, the possibility had to be eliminated that polysaccharides from medium components were contaminating the final isolated product. The modified Wickerham's medium was chosen for this purpose and was found to support excellent growth of the yeast.

In the case of the latter medium, as well as the simplified medium used in later work, the yield of crude polysaccharide was usually about one-fifth of the weight of the glucose in the medium. The polysaccharides were isolated from the supernatant of the culture in the usual manner (without concentration of the medium) and the yield was approximately 1.1 gm from 200 ml of medium. Upon acid hydrolysis followed by chromatography with the ethyl acetate-acetic acid-water solvent, three monosaccharide components could be recognized. One of these gave the R_F of xylose as well as the typical reddish-violet color that the Horrocks-Manning spray gives with a pentose. The other two spots were hexoses, one of which had the apparent R_F of mannose.

Effect of pH on polysaccharide production.—The modified Wickerham's medium was used and the pH was adjusted by the addition of K_2HPO_4 or NaOH to obtain the following: 6.4, 7.0, 7.8, and 9.0. The medium was prepared in 200 ml lots in 500 ml flasks and incubated on the rotary shaker at 30°C for 5 days. At the end of the growth period, the pH's were respectively: 5.0, 6.0, 6.3, and 8.5. Growth was poor at the acid side and excellent at pH 7 as shown in table 1. Polysaccharide yield paralleled the amount of growth. Chromatographic analysis of the hydrolyzates of the polysaccharides showed that at pH 7 and above, the typical picture of two hexose spots and the xylose spot was obtained. At the acid pH however, the slowest moving hexose spot was completely absent (i.e., the one whose R_F corresponds to glucose).

TABLE 1

*The effect of pH on growth and polysaccharide production by Torulopsis flavescens**

pH		Relative growth	Total amount of polysaccharide produced (gm)
Before inoculation	After incubation		
6.4	5.0	++	0.23
7.0	6.0	++++	2.26
7.8	6.3	+++	0.96
9.0	8.5	0	0

*Medium: Modified Wickerham's medium, 200 ml per 500 ml flask. Incubated 5 days, 30°C on rotary shaker.

Effect of carbon source on polysaccharide production.—While the modified Wickerham's medium permitted the demonstration that a pentose-containing polysaccharide was produced from glucose by the yeast, this medium was too inconvenient for further study. It was thus necessary to evolve a simpler medium of essentially synthetic composition. Through trial elimination of various components, the glucose-Casamino acids-salts medium was devised and proved to be highly satisfactory for both growth and polysaccharide production.

It next seemed interesting to determine whether polysaccharide production was dependent on the presence of glucose in the medium or whether the yeast was capable of utilizing other sugars for this purpose. The Casamino acids-salts medium was prepared in 45 ml quantities in such concentration that all the ingredients would be in their proper amounts upon the addition of 5 ml of substrate, making a total volume of 50 ml. This was contained in 125 ml Erlenmeyer flasks and incubated on the rotary shaker at 30°C for four days. The substrates used and polysaccharide yields are indicated in table 2.

Effect of glucose concentration on yield.—To study the relationship between polysaccharide yield and glucose concentration, the Casamino acids-glucose-salts medium was used and the glucose concentration was varied between 0.5 and 10 percent, but the pH was kept at 7 in all cases. The results are shown in table 3.

Properties of the polysaccharide(s).—The polysaccharide material isolated from the cultures was a white, amorphous powder which was insoluble in water but did give a rather stable, viscous suspension. It gave a reddish-brown color with iodine and a negative ninhydrin reaction, both before and after acid hydrolysis. The orcinol reaction was strongly positive while the reducing value was very low before hydrolysis and increased after heating with acid (to a degree proportional to the length of heating). The material appeared to be entirely organic (essentially no ash remaining upon ignition). In the growing culture, the polysaccharide material appeared to be a highly fibrous material. When the yield of polysaccharide in the growing culture approached the maximum, the medium became so highly viscous that it poured with difficulty.

The monosaccharides found on chromatograms of acid hydrolyzates of the polysaccharide were found to have R_F values corresponding to glucose, mannose and xylose. The identity of the latter was confirmed by elution of the spot (the edges of which were located with the detection spray) from a chromatogram developed with the ethyl acetate-acetic acid-water solvent and running the eluate on a chromatogram with water-saturated-phenol as the solvent. The unknown had the R_F of xylose with this solvent also.

TABLE 2

*The effect of varying the carbon sources on yield of polysaccharide by T. flavescens**

Carbon source	Yield of polysaccharide (gm)**
Glucose	1.96
Xylose	1.48
Mannose	2.18
Mannitol	1.16
Calcium gluconate	0.38
Galactose	1.24

*Medium: Casamino acids-salts mixture plus 0.1% KNO_3 and 1 mg/lit of thiamine HCL; pH was 7. Carbon source were autoclaved as 50% solutions and added to the basal to give a final concentration of 5%. The calcium gluconate was partly in suspension. The volume of medium was 200 ml in a 500 ml flask. Incubated 4 days at 30° C on rotary shaker.

**Chromatographic analysis of the hydrolysates of these polysaccharides showed the same three monosaccharide constituents with the exception of the polysaccharide produced from calcium gluconate, which lacked the component corresponding to glucose.

Fractionation of the polysaccharide(s).—It seemed unlikely that the polysaccharide material isolated would be homogeneous and a number of fractionation procedures were tried in order to isolate fractions of different composition. In one procedure, the material was dissolved in 4 percent NaOH, heated and centrifuged. The supernatant was then fractionated with acetone to yield two fractions, distinctly different in appearance. However, upon hydrolysis, each of the fractions yielded the same three monosaccharide components as did the unfractionated material.

The fractionation method described under Methods and Materials did, however, yield three fractions with different composition, as can be seen in table 4.

Effect of inhibitors on polysaccharide production.—1. *Fluoride.* Chung and Nickerson (1954) postulated that in the yeast they studied, polysaccharide synthesis proceeded through polymerization of glucose-1-phosphate which was produced from glucose-6-phosphate. If they added fluoride to the growth medium, phosphoglucomutase would be inhibited and polysaccharide synthesis could not occur. This inhibition could be relieved completely by the addition of a small amount of glucose-1-phosphate. In the case of *T. flavescens*, it was found that fluoride

in the concentration of $10^{-2}M$ also inhibited growth and polysaccharide production completely and the inhibition could be relieved by adding glucose-1-phosphate (0.0077M) simultaneously with the fluoride. The inhibition occurred only when the pH of the medium was on the acid side, presumably due to greater permeability of the fluoride at the lower pH. 2. *Other Inhibitors.* The effects of a number of inhibitors on polysaccharide production were determined. The concentration of inhibitor was adjusted to 0.02M with the exception of dinitrophenol which was added in a concentration of $4 \times 10^{-5}M$. The glucose-Casamino acids-salts medium was used and the pH was adjusted to 6.0 before autoclaving. The results are shown in table 5. When the concentration of an inhibitor was increased, the degree of inhibition was increased, with the exception of arsenate which was consistently stimulatory to polysaccharide production. The stimulatory effect also occurred when sucrose or maltose was substituted for glucose in the medium.

TABLE 3

*The effect of glucose concentration on polysaccharides yield by T. flavescens**

Percent of glucose in medium	Yield of polysaccharide (mg)
0.5	92.4
1.0	126.0
2.5	159.2
5.0	555.8
10.0	566.1

*Medium: Casamino acids-glucose salts; pH was 7. Incubated 5 days at 30° C on rotary shaker. Volume of medium was 50 ml in 125 ml Erlenmeyer flask.

DISCUSSION

One of the most interesting questions that arises in connection with the *Torulopsis* polysaccharides is in regard to the mechanism of synthesis of the xylose and how it is incorporated into the polysaccharide structure. Mager and Aschner (1947) suggested that hexose units might be incorporated into the polysaccharide followed by oxidation of carbon 5 and subsequent decarboxylation, yielding a pentose. However, it was not possible to detect glucuronic acid on the chromatograms under the conditions described. If the latter were present, its concentration was below that of the sensitivity of the chromatographic procedure. In addition, cell suspensions of *T. flavescens* were not able to decarboxylate glucuronic acid in manometric experiments or produce pentose from glucuronic acid. The latter does not, however, eliminate the possibility that an enzyme exists which acts on glucuronic acid when the latter is already a part of the polysaccharide. An alternative approach to the true mechanism may involve such enzymatic reactions as recently described by Avigad, Feingold, and Hestrin (1956), who were able to produce a disaccharide, xylanopyranoxyl-fructofuranoside, by incubating together xylose, raffinose, and levansucrase, or that of Putnam, Litt, and Hassid (1955), who obtained D-glucosyl-D-xylose by incubating glucose-1-phosphate, xylose, and maltose phosphorylase.

What makes it difficult to hypothesize as to the mechanism for pentose incorporation into the polysaccharides is the fact that the latter have not yet been adequately purified and characterized. If it can be found, for example, that *Torulopsis* produces a mixture of pure pentosans and pure hexosans, then it might be reasonable to suppose that there is an enzyme for polymerizing pentoses analogous to the case of hexoses. On the other hand, if the purified polysaccharide can be shown to contain alternating pentose and hexose molecules, one of the

TABLE 4

Alcohol fractionation of polysaccharides produced by T. flavescens

Fraction	Appearance	Water solubility	Yield per gm of starting material	Monosaccharides in acid hydrolyzate as shown by paper chromatography
A	yellowish-tan	insoluble	0.19 gm	glucose; trace of mannose
B	white	soluble	0.55 gm	xylose; small amount of mannose
C	white	soluble	0.04 gm	glucose; mannose and xylose in approximately equal amounts.

previously cited approaches may be valid. Further data for this point must await more adequate fractionation and purification of the polysaccharides.

The data obtained in the inhibitor experiments were the expected results; i.e., those poisons which inhibit either oxidative assimilation or respiration also inhibited growth and polysaccharide synthesis (the latter being dependent on the former, presumably). The one confusing result was the consistently stimulatory effect of arsenate on the polysaccharide production. It is well-established that arsenate can substitute for phosphate (Crane and Lipmann, 1953) in certain reactions and it would seem that arsenate is acting by inhibiting some side reaction which would otherwise remove the substrate for polysaccharide synthesis. The fact that polysaccharide production could be inhibited by fluoride and this inhibition relieved by glucose-1-phosphate would suggest that glucose-1-phosphate is an immediate precursor of polysaccharide, if the interpretation of Chung and Nickerson (1954) to this phenomenon is correct. They postulated that fluoride inhibits phosphoglucomutase, thus interrupting the conversion of glucose-6- to glucose-1-phosphate. The enzyme is presumably protected by the addition of glucose-1-phosphate.

TABLE 5

*The effect of several metabolic inhibitors on polysaccharide production by T. flavescens**

Inhibitor	Yield of polysaccharide (mg)
None	327.7
Potassium fluoride	228.3
Potassium arsenite	210.0
Sodium arsenate	433.6
Dinitrophenol	209.8
Sodium azide	170.5
Potassium cyanide	147.1

*Medium: Casamino acids-glucose-salts; pH was 6.0. Volume of medium was 50 ml in 125 ml Erlenmeyer flasks; incubated 5 days at 30°C on rotary shaker. Concentration of inhibitors was 10^{-2} M except dinitrophenol whose concentration was 4×10^{-5} M.

An important objective of this investigation of the polysaccharides produced by *Torulopsis* spp. was to evaluate the suitability of one or more of these polysaccharides as plasma volume extenders. Fraction B (see table 4) appears to be the most promising lead to such a polysaccharide in that this fraction seems to yield essentially pentose upon acid hydrolysis and represents over one half of the weight of the crude polysaccharide.

SUMMARY

Torulopsis flaveszens NRRL Y-1401, when grown aerobically, has been shown to produce high yields of polysaccharide material from sugars. The polysaccharides could be separated into three fractions which, upon acid hydrolysis and paper chromatographic analysis, yielded respectively: A, glucose with a trace of mannose and xylose; B, xylose with a small amount of mannose; C, glucose, mannose and xylose in approximately equal amounts.

Polysaccharide production and growth were optimum in a medium of approximately neutral pH. A number of sugars were satisfactory as carbon sources for polysaccharide production, with mannose and glucose giving the highest yields, respectively. Polysaccharide production could be shown to be proportional to the concentration of carbon source (glucose) in the medium with no further stimulation above 5 percent glucose. Polysaccharide production was inhibited by fluoride, arsenite, dinitrophenol, azide and cyanide but stimulated by arsenate. Fluoride inhibition could be completely reversed by the addition of small amounts of glucose-1-phosphate.

ACKNOWLEDGMENT

I am indebted to Dr. J. O. Lampen of E. R. Squibb and Sons for suggesting the problem and to the Department of Microbiology, Western Reserve University, Medical School, for making its facilities available. Sincere thanks are also due to Drs. L. O. Krampitz, Howard Gest, and G. D. Novelli of the Department of Microbiology, Western Reserve University, Medical School, for their helpful advice and comments during the progress of this work.

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