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To the Graduate Council:

I am submitting herewith a thesis written by Stephen Joseph Gagan entitled "Characterization of a Pigmented Yeast and Its Slime". I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Bacteriology.

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33

CHARACTERIZATION OF A PIGMENTED YEAST AND ITS SLIME

A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Doctor of Philosophy

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by

Stephen Joseph Gagan

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	HISTORICAL REVIEW.	2
	A. Characterization of yeast.	2
	B. Characterization of slime.	6
III.	MATERIALS AND METHODS.	10
	A. Microorganisms and culture media . .	10
	Yeast strain and stock culture . .	10
	Synthetic medium	11
	B. Procedure for the determination of the effect of carbon source.	12
	Inocula.	12
	C. Procedure for characterization of slime.	13
	Isolation and purification	13
	Preliminary analysis of intact polysaccharide	16
	Spectrophotometric analysis. . . .	17
	Hydrolysis	18
	Chromatographic procedures	18
	Identification of the components .	19

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CHAPTER PAGE

IV. RESULTS AND DISCUSSION. 20

A. Taxonomy of the yeast strain. . . . 20

B. Effect of carbon source 22

C. Characterization of slime 23

Spectrophotometric analysis . . . 23

Hydrolysis. 32

Chromatography. 35

V. SUMMARY 37

BIBLIOGRAPHY. 40

LIST OF TABLES

TABLE		PAGE
I.	The Effect of Carbon Source on Growth, Pigmentation and Slime Production. . . .	24
I.	(Continued).	25
II.	Comparison of Shaken and Stationary Cultures	26
III.	The Effect of Sugar Concentration in the Medium on Slime Production	27

LIST OF FIGURES

FIGURE PAGE

1. Infrared Spectra of Dextran, Glycogen and Yeast Polysaccharide 29

CHAPTER I

INTRODUCTION

Bacterial polysaccharides have received considerable attention because of their striking physical properties, their immunological reactions and their heterogeneity of composition and structure. The wide variety of these polysaccharides has been reviewed by Evans and Hibbert (1946).

More recently, surveys have been made of the sugar constituents of the polysaccharide fractions from a variety of fungi (Martin and Adams, 1956; Hough and Perry, 1955; Bernier, 1957; and Clutterbuck *et al.*, 1934).

Little information, however, is available on polysaccharide slimes liberated by yeasts, a property confined almost entirely to certain members of the *Cryptococcaceae*.

The nature of this problem is concerned primarily with an analysis of the slime produced by a strain of yeast. The organism itself adds to the scope of the investigation by virtue of its dark green intracellular pigment, a property which thus far lacks classification.

CHAPTER II

HISTORICAL REVIEW

A. Characterization of Yeast

This is not intended to be a digest of the current systematic classification of yeasts, inasmuch as a complete taxonomic study has been made available by the efforts of Lodder and Kreger-Van Rij (1952) and Wickerham (1951). The nature of this research, however, dictates that some attention be devoted to those aspects of yeast classification as have direct bearing on the problem.

The term "yeast" is collective in the sense that it refers to a group of fungi sharing certain outstanding characteristics. It does not, however, signify their inclusion in any one specific taxon (Cook, 1958). To the contrary, the heterogeneity of the yeasts as a group has made the confines of their domain so obscure as to often necessitate preemptory decisions with regard to the taxonomic status of any given strain.

Many consider yeasts as unicellular fungi which multiply by budding. Unfortunately, this definition applies to only a small proportion of the organisms normally classified as yeasts. Some yeasts do not multiply

by budding and most yeasts after a suitable period of cultivation will produce signs of mycelium. Skinner (1947) suggested the following definition: "Yeasts are true fungi whose usual and dominant form of growth is unicellular". The uncertainty that arises from this statement, however, is the interpretation to be given "usual and dominant growth".

Some strains of the fungi Mucor and Aspergillus, for instance, can under certain conditions exhibit a yeast-like phase. The pathogen, Histoplasma capulatum, normally shows a dominant budding phase in the host tissue (Cook, loc. cit., 1958). These organisms, however, bear little other resemblance to the yeasts. Ingram (1955) has stated that the view of the yeast as being a unicellular organism is much too restrictive.

Obviously, an irrational application of arbitrary decisions with regard to characterization of yeast can cause some confusion. However, as has been pointed out, the indecisiveness in the qualifying properties required for yeast classification is not a result of a weakness in the existing taxonomic system but can be attributed directly to the ill defined nature of the organisms themselves.

It immediately becomes apparent that anyone faced with the prospects of characterizing a previously unidentified strain of yeast might encounter some difficulty.

The task becomes insuperable if the organism exhibits properties which deviate from the normally recognized characteristics.

The literature concerning green or more generally dark pigmented yeasts is practically non-existent. This is not to infer that the existence of these forms is denied, but the principle has been not to consider the dark pigmented organisms as yeasts at all. It appears that tradition has it that only the hyaline organisms and those producing carotenoid pigments can be accepted as true yeasts, (Cook, loc. cit., 1958; Lodder and Kreger-Van Rij, loc. cit., 1952).

Harrison (1928) created two genera, Rhodotorula and Chromotorula, for the asporogenous pigment-forming yeasts. The former would include all the red pigmented organisms, the latter those other than red, eg. yellow, yellow-orange, brown and black yeasts. Lodder (1934), however, claimed that morphologically one could hardly make a distinction between the yellow, yellow-orange and red strains and proposed that they be grouped in the genus Rhodotorula. Lodder rejected the genus Chromotorula entirely. By so doing, she rejected the dark pigmented yeasts.

Mrak and Phaff (1948), exponents of the principle to consider dark pigmented organisms as "yeasts", have

suggested including the dark colored genus Pullularia in taxonomic yeast studies. Langeron (1949) also has proposed the genus Pullularia for all the dark colored strains exhibiting a yeast like phase in the early stage of growth followed by a development of a true mycelium.

A variety of black yeasts commonly isolated from dairy products have been variously named Saccharomyces niger, Torula niger, Schizosaccharomyces niger and Monilia nigra (Henrici, 1951). The latter organism described by Orla-Jensen (1902) as Cladosporium butyri has been recorded by De Vries (1952) as possessing yeast-like forms. Saccharomyces niger was referred to as an ascomycete producing spores by Marpmann (1886).

Henrici examined several of the so called black yeasts and found that they showed marked transformation of the same general principle on continued cultivation. It appears that these organisms represented a unicellular growth phase of certain of the fungi Demateaceace including Cladosporium.

B. Characterization of Slime

Many organisms are capable of producing extracellular polysaccharide material of gelatinous or gummy consistency. The material may remain firmly adherent as a discrete layer surrounding the cell, that is, as a capsule or slime layer, or the material may part freely from the cell as loose or free slime. Loose slime will normally remain as a confluent matrix around organisms in colonies on solid media but usually disperses from the cell to increase the viscosity in a liquid medium. Typical capsule forming organisms show definite capsules and according to Wilkinson (1958) always produce slime which is very similar to the capsular material. Other organisms secrete much slime but form little or no capsule (Duguid, 1951).

Two views have been advanced on the origin of the slime layer. The similarity between the chemical composition of the slime layer and that of the cell wall in few cases where both were investigated suggested the possibility of the slime layer as being a modification of the cell wall. However, in a majority of organisms studied, it has been demonstrated that the cell wall and the slime layer or capsule are quite distinct chemically. Mudd *et al.* (1943) and Etinger-Tulczynska (1931) suggested that a distinction should be made between true capsules

and extracellular slime. According to them, the true capsule is formed by a modification of the cell wall and should be considered an integral part of the organism, whereas slime represents a secretion from the cell. At present, the evidence seems to be in favor of the hypothesis of secretion, perhaps excessive secretion of cell wall material in cases where the slime and cell wall are similar or identical chemically, or secretion as a metabolic waste where no chemical relationship exists. This would apply for both the symmetrical adherent slime layer and the asymmetrical loose slime. The factor determining the physical manifestation would be the very nature of the material being secreted.

The ability to produce extracellular polysaccharides is not common to the bacteria alone, but is a property shared by molds and yeasts as well. Although polysaccharidic metabolic products of molds and yeasts are not strictly bacterial polysaccharides, they are usually considered jointly because of similarities in chemical structure (Evans and Hibbert, loc. cit., 1946).

Varianose, produced by the mold Penicillium varians G. Smith on D-glucose was found to consist of 6 to 8 β-D-galactopyranose units joined by 1:4 linkages (Haworth et al., 1935).

An immunologically active polysaccharide formed by the fungus Coccidioides immitis has been shown to consist of D-glucose, D-galacturonic acid and an unidentified nitrogen containing sugar (Evans and Hibbert, loc. cit., 1946).

Martin and Adams (1956) were able to isolate and characterize extracellular polysaccharides from 31 species of fungi representing 12 genera. Mucor and Rhizopus spp. produced material containing glucuronic acid and fucose in addition to galactose, glucose and mannose. Fusarium and Trichoderma spp. yielded glucuronic acid but not fucose. The remaining species of Penicillium, Aspergillus, Alternaria and Geotrichum produced polysaccharides lacking the uronic acid and fucose.

Bernier (1957) worked with four fungi known to be active in the decomposition of wood and forest litter. The organisms, which included Pullularia pullulans, the black mold, produced polysaccharidic slimes consisting of glucose and mannose as the major components, lesser amounts of glucuronic acid and xylose and galactose and rhamnose as trace components.

Aschner et al. (1945) demonstrated that capsules produced by the yeasts Torulopsis neoformans (Cryptococcus neoformans) and Torulopsis rotundta were composed of a pentosan plus amylose. Mager et al. (1951) tested

thirty seven different yeast species belonging to 15 genera for their ability to produce extracellular starch and found this property to be restricted to capsulated non-fermenting asporogenous yeasts. These workers concluded that on the basis of the ability of the yeasts to convert into starch organic compounds that differ widely from the initial carbon source, glucose, in their chemical structure, it can be inferred that the production of starch is in some way linked to vital metabolic processes. This conclusion was further supported by the finding that starch production by living yeasts depends on active cell proliferation. Mager et al. also observed that the growth medium of capsulated yeasts also contained, apart from starch, a soluble polysaccharide that was non-iodophylic and consisted largely of pentose. In this instance the composition of the soluble polysaccharide and the capsule was identical.

CHAPTER III

MATERIALS AND METHODS

A. Microorganisms and Culture Media

Yeast Strain and Stock Culture

The organism employed in this study was a strain of yeast isolated from rotting redwood. The yeast is exceptional in that it produces a dark green intracellular pigment and copious quantities of extracellular slime of infinite dispersion.

The organism possesses the following characteristics as applied in the current systematic classification of yeasts by Lodder and Kreger-Van Rij (loc. cit., 1952):

Growth in Malt Extract. After 3 days at 27 C, cells round to long-oval, (5-7) x (6-12) u, occurring singly, in pairs and in short chains. After ca. 30 days at 11 C, a ring and occasionally a pellicle formed.

Growth on Malt Agar. After 3 days at 27 C, cells oval to long-oval to irregular (4.5-7) x (5-10) u, occurring singly, in pairs and in chains of irregularly shaped cells. The streak culture after 20 days at 12 C rough or wrinkled, somewhat slimy or mucoid, and the color dark green to black. After ca. 3 days in liquid

and 10 days on solid media, the organism develops pseudomycelia, septate filaments arising from buds.

Fermentation. Negative.

Assimilation of Sugar. Glucose, galactose, saccharose, and maltose assimilated. Lactose not assimilated.

Assimilation of Potassium Nitrate. Positive.

Ethanol as sole source of Carbon. Growth.

Splitting of Aesculin. Positive.

Stock cultures of the organism were carried on a medium suggested by Jeanes (1956) of the following composition:

Constituent	g/l
Tryptone	2.5
K_2HPO_4	5.0
Yeast Extract	5.0
Sugar	10.0

Transfers were made weekly and cultures were maintained aerobically at room temperature.

Synthetic Medium

A medium, developed by Martin and Adams (1956) for the production of polysaccharides by molds, was found satisfactory for growth of the yeast since it yielded optimal quantities of slime and at the same time facilitated purification of the slime material.

The composition of the basal medium was as follows:

Constituent	g/l
NH_4NO_3	2.0
$K_2HPO_4.3H_2O$	1.5
KH_2PO_4	1.9
$MgSO_4.7H_2O$	0.5

The carbohydrate employed in concentrations of from 1 to 5 per cent was sterilized separately and combined with the basal medium just prior to inoculation.

The organism was maintained in an active state by continous transfers to flasks containing the liquid synthetic medium. It was found that the response of the activated cultures facilitated slime production more so than if transfers had been made from the refrigerated stock cultures.

B. Procedure for the determination of the effect of Carbon Source

Inocula

The yeast was grown in the synthetic basal medium containing 2 per cent sucrose. Cells harvested at 24 hours were washed twice with sterile distilled water and suspended in sterile 0.1 molar phosphate buffer at pH 6.8.

Duplicate 500 ml flasks containg 80 ml of the synthetic medium plus the respective carbohydrates in 2 per cent concentrations were inoculated with the above yeast suspension. One set of flasks was aerated by agitation. Both sets were maintained at room temperature. Observations for growth, pigment and slime production were made at 8 hour intervals for a period of 5 days.

Growth was measured turbidimetrically on a Klett-Summerson photoelectric colorimeter. Pigmentation was determined visually. Slime was measured by a modified Ostwald-Fenske viscosimeter.

In addition, one set of flasks was prepared with the carbohydrate concentration increased to 5 per cent. This series was aerated on the shaker. Observations were made as described above.

C. Procedure for Characterization of Slime

Isolation and Purification

Cells were grown in 500 ml flasks each containing 80 ml of the synthetic medium to which sterile sucrose had been added to give a sugar concentration of 2 per cent. It might be added here that of the various sized flasks employed, the 500 ml size gave optimal results.

Jeanes *et al.* (1957) stated that close control of conditions during dextran production was necessary to insure a product of constant properties. Martin (1958) found that in a single culture, gross differences may occur in the polysaccharide material produced by strains of *Mucor*, depending on the age of the culture. He noted that although qualitative differences were not apparent, quite pronounced changes in the relative amounts of the sugar constituents of the polysaccharide material occurred as incubation progressed. An investigation of the effect of time on the nature of the slime produced by the test yeast, however, indicated that no qualitative differences existed in the composition of the purified polysaccharide.

Subsequent cultures were harvested when slime reached maximum viscosity, usually after approximately 3 days on the reciprocal shaker at room temperature. Since the viscous nature of the cultures prevented removal of the cells by centrifugation, clarification was accomplished by suction filtration through a one quarter inch pad of Johns-Manville Celite #535 packed on circular filter paper.

The pH of the cultures at the time of harvest was usually in the vicinity of 5.5. It was found that although the maintenance of a higher pH by the addition of $CaCO_3$ resulted in a slight increase in slime production, the

difficulty that resulted in filtration obviated the advantage of the larger yield.

Attempts were made to establish the optimal pH for precipitation of the slime, the optimal being that pH which results in the maximum yield of precipitated material with the minimum concentration of contaminating substances such as inorganic salts and cellular wastes. Adjustments in pH were made with dilute NaOH and H_2SO_4 on 500 ml aliquots of culture liquid covering the range from 4.0 to 7.5 in 0.5 unit increments.

There was no change in viscosity where the pH of the culture liquor was maintained at 4.5 or above. Below pH 4.5 viscosity gradually decreased. This, apparently, was a result of molecular cleavage since on addition of 2 volumes of ethanol the resultant precipitate appeared as a light floculent mass as compared to the long, stringy material obtained at the higher levels of pH. Jeanes *et al.* (*loc. cit.*, 1957) observed a similar phenomenon with several strains of *Leuconostoc mesenteroides*. The viscosity of the cultures reached a maximum at the time of completion of dextran production and then decreased rapidly. This decrease in viscosity was attributed to partial degradation to a smaller macromolecular size, a result of aging. The yields of yeast material were identical in each case.

In consequence, no adjustments were made in pH of subsequent batches. Alternate solution of the gray white stringy precipitate in water and precipitation with cold ethanol was repeated several times. The precipitate was washed with ethanol and then repeatedly with ether and dried under vacuum. A yield of 1.5 gm could be obtained from 5 liters of culture.

Preliminary Analysis of Intact Polysaccharids

Total nitrogen was determined by semi-micro Kjeldahl procedure.

Total organic phosphorous was determined by the method of Goodwin *et al.* (1958).

Non-carbon ash was determined by the procedure recommended for analysis of wheat flour in the Association of Official Agricultural Chemists publication.

Total reducing activity was measured by the anthrone procedure described by Mokrasch (1954).

Semi-micro determination of uronic acid (Maher, 1949) is based on the fact that when a uronic acid is heated with hydrochloric acid, it is decomposed to furfural, carbon dioxide and water, and the yield of carbon dioxide is quantitative.

Spectrophotometric Analysis

Absorption spectra were obtained on the polysaccharide in the ultraviolet region on a Beckman Model D. U. Spectrophotometer and in the infrared on a Perkin-Elmer Model 137 Infrared Spectrometer. Samples prepared for ultraviolet analysis consisted of various dilutions of the purified material dissolved in distilled water. The potassium bromide pellet method of Schiedt and Reinwein (1954) and by Stimson and O'Donnel (1952) was used in the preparation of samples for infrared spectroscopic examination.

In the preparation of the pellets, 198 mg of dried and ground spectrographic grade potassium bromide was ground with 2 mg of the sample. The mixture was transferred quantitatively to a pellet die (Hilger and Watts Ltd.), evacuated for 5 minutes and subjected to 20,000 pounds pressure under vacuum for 5 minutes in a Carver press. A plain potassium bromide pellet was used as reference.

The Infrared Spectrometer was adjusted to 90 per cent transmittance at 4.5 microns set for automatic slit width adjustment and run at a speed of 1 micron per minute.

Hydrolysis

Hydrolysis of the purified polysaccharide was attempted by several methods. Solutions of sulfuric or oxalic acids were used with varying times and temperatures. Hydrolysis in the presence of cation exchange resin (Dowex 50) and by enzymatic action (Takamine Cellulase and Pectinase) were also tried.

Acid hydrolysates were neutralized with barium hydroxide and the insoluble barium salt of the acid was removed by centrifugation. Solutions prepared in this way were used for chromatographic analysis.

The extent of hydrolysis was measured by determining the change in reducing activity using the Folin-Wu method for colorimetric blood sugar analysis.

Chromatographic Procedures

For paper chromatography, a 7 x 24 glass cylinder was used. Samples of the hydrolysate were applied to Whatman #1 filter paper strips with a micro pipette and dried on a glass plate warmed over a hot water bath. The strips were suspended in the cylinder for descending irrigation, equilibrated with the solvent and the cylinder was then sealed with a plate glass cover. Acidic (ethyl acetate, acetic acid and water in a ratio of 3:2:1.5) and neutral (butanol, ethanol and water in a ratio of 4:1:5)

solvents were used. For the detection of reducing compounds, aniline hydrogen phthalate and p-anisidine hydrochloride (3 per cent in ethanol) were used. The o-phenylene-diamine reagent of Deley (1954) was employed for the detection of phosphate esters.

The movements of the unknown samples were compared with those of known compounds for which Rf values had been determined. In addition, known compounds were run simultaneously with the unknowns in order to compensate for deviations resulting from uncontrollable changes in test conditions.

Identification of the Components

A variety of tests were employed to identify the components released by hydrolysis. In addition to the chromatographic procedure already presented, the following were included: ninhydrin for amino sugars, Dische's carbazole reaction for hexuronic acids, (1947), Tauber's test for pentoses and Saliwanoff's test for keto sugars.

Wherever possible, phenylhydrazine dervatives were attempted. The time of osazone formation and the melting points of insoluble osazones were established (Hassid and McCready, 1947).

CHAPTER IV

RESULTS AND DISCUSSION

A. Taxonomy of the Yeast Strain

The exact taxonomic position of the yeast strain was not determined. According to Lodder and Kreger-Van Rij (loc. cit., 1952), yeasts can be divided into three major groups:

a. Endomycetaceae. The ascosporogenous yeasts.

b. Sporobolomycetaceae. Yeasts which form ballistospores.

c. Cryptococcaceae. The asporogenous yeasts which form neither asci nor ballistospores.

The test organism has never been observed to produce ascospores or ballistospores. This would tentatively establish the strain as a member of the Cryptococcaceae. However, no place has been reserved in this group for the dark-colored strains; only hyaline organisms and those producing carotenoid pigments are accepted (Cook, loc. cit., 1958).

A fact worthy of consideration is that yeasts are subject to the same phase variations that occur in bacteria. In addition to changes in morphological properties, biochemical transformations may also take

place. This might result in variation or total loss of pigment, spore forming variants in what were initially asporogenous strains or the reverse occuring where ascosporogenous strains would be depleted of their sporulating property (Cook, loc. cit., 1958; Fabian and McCullough, 1934).

The existence in nature of dark pigmented organisms analogous to non or light-pigmented yeasts is recognized. However, apart from their being in existence, no concerted effort has been made to categorize them--to the contrary, they seem to be regarded as a legion of anamolous outcasts. The fact is that the tendency has been not to consider dark pigmented organisms "yeasts" at all. It appears "tradition" has it that only hyaline organisms and those producing red or yellow pigments are accepted among the yeasts (Lodder and Kreger-Van Rij, loc. cit., 1952).

This apparent weakness in our taxonomic scheme is unfortunate, but lends credence to the oft stated opinion that no clearly defined groups of yeasts have as yet been found to occur in nature (Cook, loc. cit., 1958).

B. Effect of Carbon Source

The versatile nature of the yeast strain is indicated by its ability to utilize a wide variety of carbon sources not only for growth but for pigment and slime production as well (Table I). The data is even more significant when one considers that the basal medium is in many respects one of minimal nutritional value.

The failure of the organism to use the Krebs' cycle intermediates, with the exception of succinate, was at first interpreted to signify an absence of the tricarboxylic acid system. Krebs (1943) was of the opinion that yeasts lacked this major oxidative pathway since he was never able to find any of the intermediates in the medium. This response, however, is due to the impermeability of the cell membrane to these acids (Cook, loc. cit., 1958).

One feature is evident from the table. Conditions optimal for growth, pigment and slime production are not necessarily related. The evidence of pigment only under highly aerobic conditions might imply a possible oxidative or respiratory role in the metabolic processes of the organism. Unfortunately, the pigment is maintained within the cell and the customary techniques for the

extraction of intracellular components of yeast cells proved unsuccessful (Peterson and Bell, 1953).

Another fact worthy of mention is the ability of the organism to produce slime in shake and still cultures in almost equivalent amounts even though shake cultures produced more cells and utilized more carbohydrate (Table II). A similar observation was made by Haynes (1951) for Pseudomonas aeruginosa and by Eagon (1956) for P. fluorescens. It can also be observed that a somewhat constant maximal concentration of slime is produced if the concentration of sugar in the medium is maintained above 2 per cent (Table III).

C. Characterization of Slime

Spectrophotometric Analysis

Studies of the region 960-760 cm^{-1} in the infrared spectra of a large number of glucopyranose derivatives has revealed that absorption peaks in this region can be correlated with certain stereochemical features in the molecules (Orr, 1954; Barker et al., 1956). Perhaps the most significant relationship to evolve from these studies is that all those derivatives having the α-configuration absorbed at ca. 844 cm^{-1}, whereas, a β-configuration absorbed at 891 cm^{-1}. Additional studies

TABLE I

THE EFFECT OF CARBON SOURCE ON GROWTH, PIGMENTATION AND SLIME PRODUCTION

Carbon Source	Growth	Pigment	Slime
D-glucose	+	+	+
D-galactose	+	+	+
D-mannose	+	+	+
D-fructose	+	+	+
D-arabinose	+	+	+
L-rhamnose	+	-	-
D-ribose	+	-	-
D-xylose	+	+	+
Dulcitol	-	-	-
Mannitol	+	-	+
Sorbitol	+	+	+
Glycerol	+	+	+
D-Galacturonic acid	-	-	-
D-glucuronic acid	-	-	-

TABLE I

THE EFFECT OF CARBON SOURCE ON GROWTH, PIGMENTATION AND SLIME PRODUCTION (CONTINUED)

Carbon Source	Growth	Pigment	Slime
Sucrose	+	+	+
Maltose	+	+	+
Lactose	-	-	-
Sodium acetate	+	+	+
Starch	-	-	-
Glycogen	-	-	-
Yeast polysaccharide[a]	-	-	-
Citrate	-	-	-
Fuma rate	-	-	-
-Ketoglutarate	-	-	-
Malate	-	-	-
Lactate	-	-	-
Succinate	+	±	-
Oxalate	-	-	-

[a]purified form

TABLE II

COMPARISON OF SHAKEN AND STATIONARY CULTURES

Cultures	Glucose utilized, g/l	Growth, O. D.	Polysaccharide g/l
Shaken	18.5	1.8	0.38
Stationary	11.0	0.9	0.36

TABLE III

THE EFFECT OF SUGAR CONCENTRATION IN THE MEDIUM ON SLIME PRODUCTION

Concentration of Sugar in Medium Per Cent	Polysaccharide produced, g/l
0.5	0.24
1.0	0.30
1.5	0.30
2.0	0.36
3.0	0.39
4.0	0.41
5.0	0.39

have also revealed that in so far as polymer identification is concerned, α-polyglucosans of the starch and glycogen class (1:4 linkages) absorb at 930 ± 4 and 758 cm^{-1}, whereas, those of the dextran class (1:6 linkages) absorb at 917 ± 2 and 768 cm^{-1}.

The infrared spectra of two glycosidic polymers, dextran and glycogen and the yeast polysaccharide are compared in Figure I. Absorption peaks at 930 and 758 cm^{-1} definitely establish the yeast polysaccharide as having the α-configuration and that it belongs to the starch class with a predominantly 1:4 linkage. These peaks are possibly not as sharp as might have been expected, but this undoubtedly can be attributed to basic impurities associated with the preparations.

The absorption of the yeast polysaccharide at *ca.* 1610 cm^{-1} is somewhat perplexing. Strong peaks at 1600 cm^{-1} normally denote the carboxylate ion arising from a compound such as uronic acid (Quinell *et al.*, 1957; Orr, 1954; Burket *et al.*, 1952). In accord with this is the unusually high ash content of the polysaccharide (16 per cent). As salts of uronic acids, metal ions would be removed only with difficulty. The results of both qualitative and quantitive tests on the intact polysaccharide and on acid hydrolysates, however, failed to demonstrate any uronic acid residues.

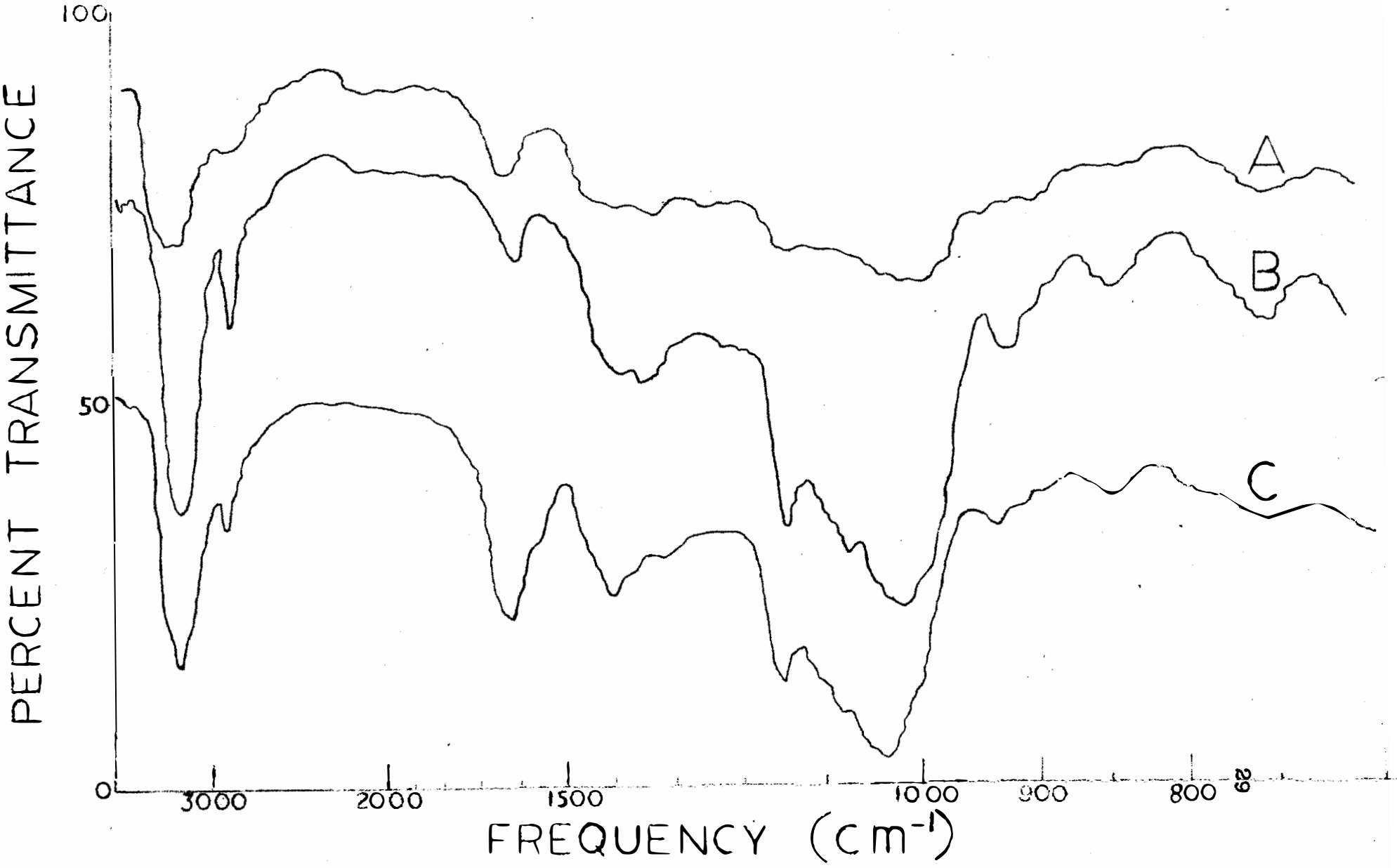


Figure 1. Infrared spectra of: (A) dextran; (B) glycogen; and (C) yeast polysaccharide.

A second alternative exists which might account for the absorption in this range and that is the presence of water (Lawson and Stacey, 1954). Preparatory to ashing, it was first necessary to establish the moisture content of the yeast polysaccharide. The average value obtained was 11 per cent. This, it is believed, would be sufficient to establish a significant peak. It was considered unwise, however, to make any attempt to remove this moisture by exposure to high temperatures as this might have resulted in some destruction of the polymer.

The similarity between glycogen and the yeast polysaccharide might stimulate speculation as to the possible role of the glycosan as reserve carbohydrate. It was found, however, that the polysaccharide does not appear to be utilized by the organism in the purified form. This may be a result of the alteration of the material during the isolation and process of purification from its composition in the "native" state. It is doubtful, however, whether any alteration actually occurs; for example, the purified polysaccharide of the pneumococci will react with antibodies produced by whole cells (Dubos, 1949). It is possible that the reason for the lack of utilization is that the

polysaccharide does not function as a storage product, but rather as an accumulation of waste products. Dubos (1949) has suggested that capsules and slimes are waste products. He sustains his view by pointing out the results of studies concerning the metabolic origin of dextrans and levans of certain bacteria, in which high molecular weight polysaccharides are formed from the residue of the carbohydrate substrate which the organism does not utilize.

The inability of the yeast to utilize the polysaccharide is further emphasized by the absence of any extracellular glucosidic enzymic activity. In view of the source of the organism, cellulolytic activity might have been expected.

Although microorganisms vary in their carbohydrate synthesizing powers, polysaccharides of different degrees of complexity can be found in nearly all bacteria and related organisms (Porter, 1948). The polysaccharide may be composed solely of units introduced in the substrate, of these units, or related compounds found in combination with unrelated substances. This heterogeneity has made it difficult to establish one procedure applicable in the complete analysis of all types of polysaccharides. The method ultimately chosen for any given glycoside is

usually one resulting from a series of trial and error experimentation.

The purified polysaccharide contained approximately 16 per cent non-carbon ash, 0.8 per cent phosphorous, and 1.9 per cent nitrogen which was not present as an amino sugar constituent. A number of bacterial polysaccharides have been reported to contain up to 1 per cent non-amino sugar nitrogen, the removal of which is very difficult and usually involves degradation of the polysaccharide. Stacey (1954) has suggested that this nitrogenous constituent represents the "vestigial remains" from nucleoprotein of the enzymes responsible for synthesis of the polysaccharide. An absorption spectrum in the ultraviolet region indicated the absence of protein and nucleic acid.

Hydrolysis

To determine the maximum theoretical reducing activity present in the polysaccharide, the anthrone procedure was used. The value of 79 per cent reducing sugar expressed as glucose was found by comparison with standard glucose curve.

Three separate approaches were considered in attempts to hydrolyze the polysaccharide produced by this yeast. These techniques differ in the ultimate effect they may have on the constituents as they are released from the polymer. Any alteration or destruction of these constituents and the degree of change would be dependent on the severity of the hydrolytic process and on the stability of the constituents themselves (Quinell *et al.*, 1957).

To illustrate, when a furanose of a sugar exists in a glycoside, the linkage is split easily and only mild acid and heat treatment is required. Pyranosides, however, are able to survive this mild treatment and their complete degradation may require 2 to 5 per cent acid at 120 C for several hours during which time destruction of some constituents such as uronic acids may occur. Quite often, this problem can be resolved by a partial hydrolysis with weak acid solution followed by complete degradation by the more drastic conditions. This method obviously can be only qualitative at best.

The polysaccharide appeared to be partially resistant to acid hydrolysis. Using a 1 N sulfuric acid solution, a reducing value of 43 per cent was obtained after 1 hour at 120 C. After 4 hours, the

value had reached only 52 per cent of theoretical. Approximately 85 per cent of the theoretical reducing activity could be obtained but only after 24 hours of hydrolysis under the above conditions. Apparently, during the initial stages partial hydrolysis had occurred which succeeded in cleaving branch units, leaving a limit dextrin type core which resisted hydrolysis. This reasoning appears to be in line with the effect pH was observed to have on the viscosity of culture liquor and the nature of the precipitate when the pH was lowered below 4.5.

It was found that the polysaccharide could also be hydrolyzed in water in the presence of approximately 10 times its weight of a cationic exchange resin (Dowex 50). Since the resin was available only in the sodium form, acid regeneration was accomplished by suspending the resin in 5 per cent H_2SO_4 for 1 hour. The resin was washed with several volumes of distilled water until the pH of the washings remained constant at pH 6. Hydrolysis was best accomplished in sealed pressure bottles at 100 C for 36 hours.

Enzymatic hydrolysis was also attempted employing commercial preparations of cellulase. Reducing activity was obtained but only to the extent of 42 per cent of

theoretical. As a result, only qualitative analytical determinations were made on constituents released by enzymic action. This milder form of hydrolysis served as an indicator of possible destruction of components by the more destructive acid hydrolysis.

Chromatography

To determine the products of hydrolysis, paper chromatograms were made of the acid and cationic resin hydrolysates during various stages of treatment. In every case, only one spot with an R_f of 0.34 was revealed when developed with aniline phthalate reagent. This spot was identified as glucose.

The hydrolysates were ninhydrin negative indicating the absence of amino sugars. Tauber's phloroglucinal test for pentoses and Saliwanoff's test for keto-sugars were negative. In addition, Dische's carbazole reaction for hexuronic acids was negative and Kapp's procedure for the microestimation of uronic acids in the unhydrolyzed polysaccharide was also negative.

Reaction of phenylhydrazine with hydrolysate resulted in a precipitate which formed in approximately 5 minutes. The insoluble osazone gave a melting point

of 202 - 205 C. Both the time of formation and the melting point of the osazone are characteristic of glucose.

CHAPTER V

SUMMARY

The organism employed in this study was a strain of yeast isolated from rotting redwood. It produces an unidentified dark green intracellular pigment and copious quantities of extracellular polysaccharide slime of infinite dispersion.

Slimes were produced from culture media containing such varied carbon sources as hexoses, pentoses, disaccharides, sugar alcohols, glycerol and sodium acetate. Stationary cultures yielded slime in amounts equivalent to that obtained by shaker cultures, although the latter produced more cells and pigment.

The purified polysaccharide contained ca 1.9 per cent N, 0.8 per cent P and 16 per cent non-carbon ash. Ultraviolet analysis indicated the absence of protein and nucleic acid. The anthrone carbohydrate test indicated 79 per cent reducing activity when expressed as glucose.

Infrared spectrophotometric analysis produced a spectrum resembling that of glycogen. An absorption peak at 840 cm^{-1} indicated an α-configuration whereas absorption peaks at 930 cm^{-1} and 758 cm^{-1} suggested a

predominantly 1:4 linkage. This is in contrast to typical bacterial dextrans which possess 1:6 linkages.

Hydrolysis of the polysaccharide was accomplished with 1 N sulfuric acid at 120 C for a period of 24 hours. The hydrolysate was ninhydrin negative indicating the absence of amino sugars. Dische's carbazole reaction for hexuronic acids was negative and Kapp's method for the microestimation of uronic acids in the unhydrolyzed polysaccharide was also negative. In addition, Tauber's test for pentoses and Saliwanoff's test for keto sugars were negative.

Time of osazone formation and melting point determinations verified the sugar as glucose.

Analysis of the slimes produced from four other carbon sources, e. g. mannose, glucose, xylose and glycerol indicated that their composition was identical.

If one disregards the difference in the physical manifestation of the extracellular material, a relationship may exist between our yeast and members of the Cryptococcaceae. The starch-like capsule produced by Cryptococcus neoformans exhibits a preponderance of α-links and has been classified as an unbranched 1:4 glucosan. Some of the members of the genus Cryptococcus are known to secrete a starch-like compound into the

medium but only under appropriate conditions of pH. The fact that no place has been allowed the dark pigmented yeasts in the family Cryptococcaceae, however, prevents definite assignment of this organism.

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