UDC 582.282.23 : 581.19 : 547.45

11. Fusaé Kogané and Tomomichi Yanagita : Qualitative and Quantitative Studies on the Cell Wall Carbohydrate in the Yeast, *Schizosaccharomyces pombe*.

(*Institute of Food Microbiology, Chiba University*[*1])

On studying the mechanism of biosynthesis of cell wall substance, such a microörganism which is constructed with a cell wall of simple constitution may be desirable for the test organism. As reported by Cook,[1] the yeast, *Schizosaccharomyces pombe*, was found to have a cell wall of simple carbohydrate composition, i. e., glucose alone. Recent investigation of Nagasaki and Yamadaira,[2] however, showed that the cell wall carbohydrate of this yeast consisted of glucose, mannose and galactose.

Present investigation confirmed the observation presented by Cook that the cell wall carbohydrate of this yeast consists of glucose alone. Further experiments were performed to establish the method of quantitative determination of cell wall carbohydrate and the method was applied to the comparison of cell wall carbohydrate content in cells grown in the absence and presence of anti-yeast antibiotics.

Experimental

Cultivation of Test Organism——*Schizosaccharomyces pombe* strain N. C. Y. C. 132 (supplied by courtesy of Dr. Mitchison of Edinburgh University) was cultured with shaking in the modified Cza pek-Dox medium of the following composition : yeast extract, 2.0 g.; $(NH_4)_2SO_4$, 2.0 g.; glucose, 20.0 g.; K_2HPO_4, 1.0 g.; NaCl, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; water to 1000 cc. (adjusted to pH 5.0). One day culture of this yeast was used as inoculum.

Isolation of Cell Wall Preparation for Qualitative Analysis——Cells grown in 150 cc. of medium were harvested by centrifugation and washed twice with dist. water. These cells were further treated as shown in Chart 1. Cells suspended in water were subjected to sonic disintegration with glass beads (average diameter : 0.3 mm.) by magnetostriction oscillator (10 Kc.) for 30 min. Almost complete disintegration of cells was checked by microscopical examination. After several washings of the sonicate, the residue was incubated overnight at 37° with buffered solution of crystalline trypsin (" trypsillin " of Mochida Pharmaceutical Co., 4,000 HUM per tube) overlayered with toluene. The white turbid hydrolysate thus obtained was washed exhaustively with water and organic solvents, and the residue was used as the cell wall preparation. This was then subjected to qualitative analysis.

Cell Suspension
↓ sonication (with glass beads), 30 min.
Sonicate
↓ decantation (removal of glass beads), centrifugation (10,000 r.p.m.), 10 min.
Ppt.
↓ wash three times with water, trypsin treatment (37°, 1 day), centrifugation (10,000 r.p.m.), 10 min.
Ppt.
↓ wash three times with water, wash once with ethanol, wash once with ether
Ppt.
(Cell Wall)

Chart 1. Flow Sheet of Preparation of Cell Wall

*1 Okubo, Narashino, Chiba-ken (小金芙紗江, 柳田友道).

1) A. H. Cook : " The Chemistry and Biology of Yeasts," 238 (1958), Academic Press, New York.
2) K. Nagasaki, T. Yamadaira : Paper presented at General Meeting of Agricultural Chemical Society of Japan, Tokyo (1959).

Qualitative Analysis of Cell Wall Components——Hydrolysis of the cell wall preparation was performed mostly by the method of Whistler and Smart.[3] The cell wall was dissolved almost completely in 75% H_2SO_4, and the solution was diluted with dist. water to make the final concentration of H_2SO_4 to 1*N*. It was then heated in a boiling water bath for 3 hr. under reflux. The duration of hydrolysis was preliminarily checked to be enough as shown in Fig. 1, although the hydrolysis was incomplete. The hydrolysate was treated with $BaCO_3$ to remove SO_4^{2-} and the resultant solution was concentrated *in vacuo* to a small amount. This was subjected to paper chromatography to analyze the carbohydrate composition.

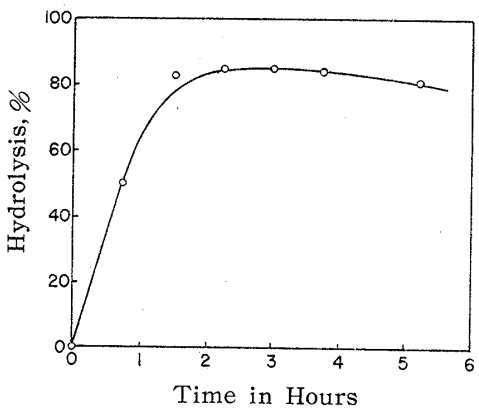

Fig. 1. Time Course of Acid Hydrolysis of Cell Wall Preparation

Cell wall sample was prepared by the method of Bell and Northcote.[6] For conditions of hydrolysis see text. Percentage hydrolysis is expressed in terms of amount of glucose formed per dry weight of cell wall preparation.

Estimation of Reducing Sugar and Nitrogen Contents——The method of Folin and Malmros[4] was used for the estimation of reducing sugar content in an aliquot of the hydrolysate of cell wall preparation, and the method of Akamatsu[5] for nitrogen content of a Kjeldahl digest of an aliquot of whole cell suspensions.

Materials——The anti-yeast antibiotics used were as follows : crystalline cycloheximide (obtained by courtesy of Tanabe Pharmaceutical Co.), trichomycin (3460 u./mg.), crystalline aureothricin, nystatin, and variotin. The latter four antibiotics were supplied by courtesy of Japan Antibiotics Research Association.

Results and Discussion

Carbohydrate Component of Cell Wall Preparation

Two dimensional paper chromatography of the acid hydrolysate of isolated cell wall preparation gave only one spot as visualized by coloring reagents, such as ammoniacal silver nitrate, anisidine reagent and Ninhydrin (to detect amino sugar). Rf-values of the sample for two solvents used are given in Table I as compared with those of authentic sugar samples taken as reference standards. Since Rf-values of glucose, mannose and galactose are quite similar, the other solvent systems were then tested. It was, however, found to be difficult to separate these sugars distinctively. Therefore, the phenylhydrazine derivative of the sample was compared with those of the 3 sugars.

The sample was mixed with an acetic acid solution of phenylhydrazine and kept at 4°. Under the same conditions, mannose formed fine light yellow crystals of m.p. 179°, while the sample remained unchanged. When the mixture was heated in a boiling water bath for 45 minutes, an abundant formation of yellow needles (m.p. 203° after recrystallizations from ethanol) was observed. The same experiments were carried out using glucose and galactose. The former gave crystalline osazone of m.p. 203° and the latter that of m.p. 178° (decomp.). From these observations the major component of the cell wall carbohydrate was identified to be glucose (mixed melting point of the derivative of the sample with that of glucose remained undepressed).*2

*2 Major part of this wall preparation was considered to be composed of "yeast glucan," since cellulose reactions were negative.

3) R. L. Whistler, C. L. Smart : "Polysaccharide Chemistry," 55 (1953), Academic Press, New York.
4) O. Folin, H. Malmros : J. Biol. Chem., **83**, 115 (1929).
5) S. Akamatsu : J. Biochem. (Tokyo), **39**, 203 (1952).

Amino acid composition of the cell wall hydrolysate was also studied. Faint Ninhydrin-positive spots of glycine, alanine and other small number of unidentified amino acids were so far detected.

It should be mentioned that some part of cell wall preparation resisted the hydrolysis with 1*N* sulfuric acid. Thus, after the acid hydrolysis of such cell wall preparation, flocculent precipitates remained in the hydrolysate. These precipitates were collected, washed, and subjected to a drastic hydrolysis with 6*N* hydrochloric acid for 2.5 hours in a boiling water bath. This treatment resulted in the dissolution of almost all the precipitates. The hydrolysate thus obtained was evaporated and paper-chromatographed with a solvent system of butanol-acetic acid. The chromatogram showed 5 distinctive Ninhydrin-positive spots of low Rf-values (0.19, 0.17, 0.095, 0.058, 0.027) and no spots positive to ammoniacal silver nitrate reagent. These results suggest that the part of cell wall preparation resistant to 1*N* sulfuric acid-hydrolysis is not polysaccharide but polypeptide.

Table I. Rf-values of Cell Wall Hydrolysate and Various Carbohydrates used as Reference Standards

Sugar	Solvent System	
	PhOH-H_2O (4:1)	BuOH-AcOH (25:3)
Glucose	0.41	0.17
Mannose	0.45	0.19
Raffinose	0.35	0.03_8
Galactose	0.45	0.11_5
Glucosamine	0.64	0.10
Inositol	0.22	0.04_5
Cell wall hydrolysate	0.42	0.20

Method of Quantitative Estimation of Cell Wall Carbohydrate

In the isolation of the cell wall fraction of cells as shown in Chart 1, a partial loss of the cell wall fraction through repeated centrifugations is highly probable. Therefore, the reproducible quantitative isolation of the cell wall fraction may not be expected by the method presented in the Chart. In contrast to this mechanical method, Bell and Northcote[6] presented a chemical method of isolating cell wall fraction in yeast (Chart 2). Although the latter method seems to meet more likely the present aim of quantitative isolation than the former, it seems to be still too much complicated for this purpose. Therefore, this chemical method was examined to apply to the quantitative isolation. Thus, dry weight, carbohydrate and nitrogen contents of a sample of each step numbered

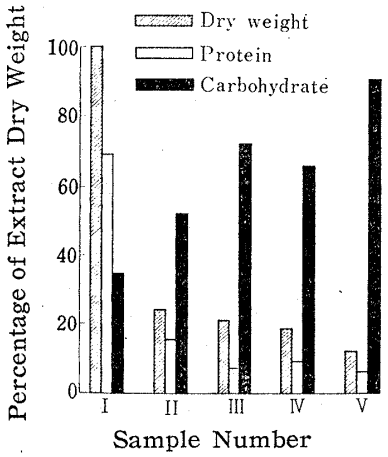

Fig. 2. Carbohydrate and Protein Contents in Successive Samples of Cell Wall Extraction by the Method of Bell and Northcote[6]

Numbers on abscissa express numerical order of extraction of cell wall (see Chart 2). Shaded bar represents percentage of dry weight of each extract per that of initial cell sample (I). White and black bars denote percentages of protein and carbohydrate contents, respectively, per dry weight of each extract. Protein factor, 6.45.

6) D. J. Bell, D. H. Northcote : J. Chem. Soc., **1950**, 1944.

successively in Chart 2 were measured for the comparison. As shown in Fig. 2, percentage of nitrogen in a sample of each step decreased as the process of extraction proceeded, while that of carbohydrate increased reaching an apparent plateau at step III. These results suggest that the extraction of cell wall fraction is almost complete and sufficient for the quantitative analysis at step III. Therefore, in the following experiments, the extraction procedure was terminated at step III in Chart 2 followed by successive washings with 2% acetic acid and distilled water.

I. Cells
| 6% NaOH, heat at 60°, dilute 8-fold
II. Ppt.
| 3% NaOH, room temperature, 3 hr., dilute 3-fold
III. Ppt.
| water, heat at 80°, pH to 4.5
IV. Ppt.
| 3% NaOH, heat at 80°, 2 hr., centrifugation, water, pH to 4.5, centrifugation, wash with 2% AcOH
V. Ppt.
(Cell Wall)

Chart 2. Flow Sheet of Chemical Method of Isolation of Yeast Cell Wall after Bell and Northcote[6]

Change in Cell Wall Carbohydrate Content in Cells during Cultural Development

It has been known that change in the amount of cell wall of microörganisms generally depends upon the duration of culture.[7] This was examined with this fission yeast as a preliminary experiment of the following investigation. As given in Table II, cells harvested at various phases of cultivation were compared for their total nitrogen and carbohydrate content of the wall substance. The total nitrogen content of cells seemed to decrease as growth proceeded. This was confirmed by repeated experiments. Not so much difference in the amount of wall substance (carbohydrate content) was observed among those cell samples.

Table II. Change in Total Nitrogen Content and Amount of Cell Wall Substance in Cells of Different Ages

Time of Cultivation (hr.)	Number of Cells[a] (cells/cc.)	Total Nitrogen Content (% dry wt. of cells)	Cell Wall Carbohydrate Content (% dry wt. of cells)
18.0	8.4×10^5	13.8	18.0
29.5	8.0×16^6	12.0	17.9
65.0	4.2×10^7	12.1	18.1

a) Total number of cells in a culture was counted by the use of Coulter's blood cell counter and cell size analyzer.

Comparison of Cell Wall Carbohydrate Content in Cells grown in the Absence and Presence of Antibiotics

It may be probable that those antibiotics specifically active against yeasts but not against bacteria inhibit the formation of cell wall materials of the yeast cells, since the cell wall components of yeasts and bacteria are quite different. From such a consideration, the cell wall carbohydrate contents in cells grown in the absence and presence of some antibiotics at an inhibitory concentration were compared to find out antibiotics which suppress the biosynthesis of the cell wall carbohydrate.

7) G. D. Shockman, *et al.*: J. Biol. Chem., **230**, 961 (1958).

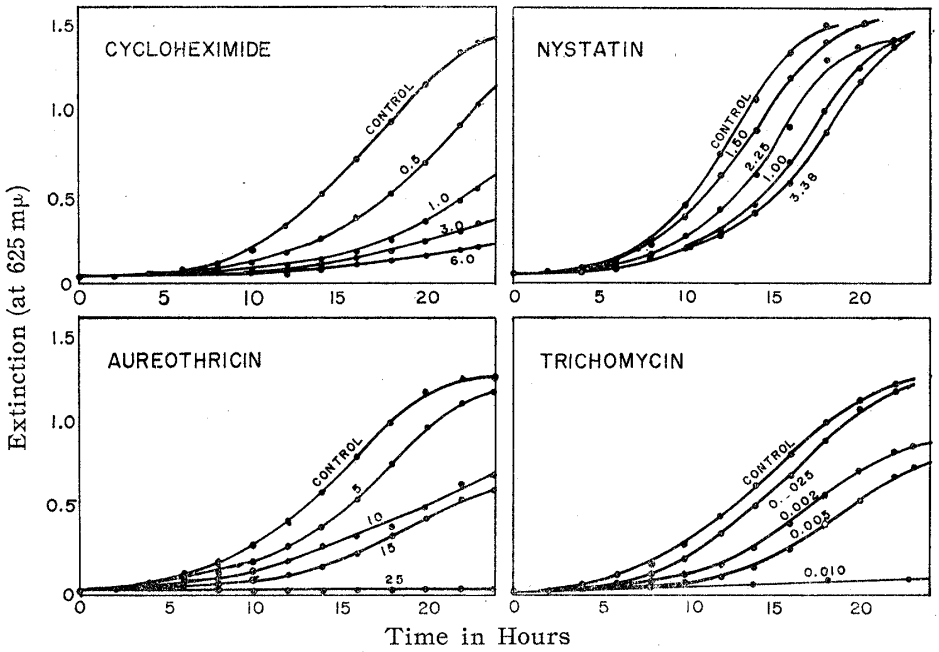

Fig. 3. Growth Curves of *Schizosaccharomyces pombe* in the Presence and Absence of Graded Concentrations of Anti-yeast Antibiotics

Extinction was measured photometrically using Coleman Universal Spectrophotometer Model 14. Figures indicated are concentrations of antibiotics expressed in terms of γ./cc. Effect of variotin on growth was not followed because of appearance of turbidity caused by incomplete dissolution of this antibiotic in this medium.

Table III. Total Nitrogen and Cell Wall Carbohydrate Contents of Cells grown in the Presence and Absence of Inhibitory Concentration of Various Anti-yeast Antibiotics

Antibiotics	Concentration of Antibiotics (γ/cc.)	Growth Inhibition[a] (%)	Total Nitrogen (% dry wt. of cells)	Cell Wall Carbohydrate (% dry wt. of cells)
Control			10.5	17.4
			9.9	21.5
			11.2	20.3
Cycloheximide	1.13	52	9.6	19.5
	1.13	24	9.0	17.8
	1.33	34	10.2	18.2
Nystatin	1.60	65	10.3	20.3
	1.60	41	11.5	21.0
	2.00		10.9	19.0
Aureothricin	4.4	53	11.3	23.1
	4.4	75	12.1	15.2
	4.4	88	14.4	19.2
Trichomycin	0.0053	84	9.1	16.7
	0.0053	78	11.9	21.1
	0.0053	91	14.7	17.5
Variotin	3.33	90	13.3	18.5
	3.00	92	13.6	22.7
	3.00	44	11.3	20.7

a) Degree of growth inhibition was expressed in terms of percentage of dry weight/cc. culture of inhibited cells to that of control cells.

Prior to this experiment, the growth of this yeast in the absence and presence of graded concentrations of antibiotics was followed using Coleman spectrophotometer. Fig. 3 shows the data obtained for each antibiotic. Thus the adequate concentration of each antibiotic for the experiment designed above was determined. The time of harvest of cells was also determined as 17th hour of cultivation. At this time, cultures were in the logarithmic phase of growth in almost all the cases. Thus the wall carbohydrate content of cells was compared at steadily growing condition.

In Table III, data of total nitrogen and the cell wall carbohydrate content of cells grown in the absence and presence of various antibiotics are listed. In each case triplicate experiments were run in separate dates, since some degree of variance in data was found to be inevitable in such experiments. As may be deduced from these figures shown in this table, no antibiotics so far tested appeared to affect the cell wall carbohydrate content of the yeast cells.

The authors express their gratitude to Tanabe Seiyaku Co. and Mr. Y. Yagisawa of Japan Antibiotic Research Association for supplying various antibiotics.

Summary

The cell wall sample isolated from the mechanically disintegrated cells of the fission yeast, *Schizosaccharomyces pombe*, was found to be composed of glucan as a major component accompanied with a small amount of peptides. For the quantitative estimation of the wall carbohydrate, the chemical extraction method of Bell and Northcote was employed with slight modifications. The wall carbohydrate content in cells of different ages and in those grown under the influence of anti-yeast antibiotics was estimated.

(Received January 6, 1961)