OBSERVATIONS ON CELL WALLS OF YEASTS AND SOME OTHER FUNGI BY X-RAY DIFFRACTION AND SOLUBILITY TESTS

by

D. R. KREGER

Laboratory of Technical Botany and X-ray Department of the Laboratory of Technical Physics, Technical University, Delft (Netherlands)

INTRODUCTION

In the study of cell walls of yeasts and other micro-organisms, chemically intact, clean cell wall material is a first requirement. The usual method of obtaining the cell wall material, by an alkali digestion of the whole yeast, involves insufficient removal of the cell contents, and the cell walls are chemically attacked. These drawbacks have restricted cell wall studies of yeasts and other fungi.

Some years ago suitable apparatus became available for the mechanical separation of the cell wall of yeasts from its contents[1]. This has recently led to chemical, electron microscope and X-ray diffraction studies[2,3,4,5] of mechanically isolated and, hence, chemically intact cell walls. The work mainly concerned baker's yeast, although observations on other species have been included[2,4]. As a result of this work it has been confirmed that certain yeast constituents isolated before from the whole yeast are definitely part of the cell wall. These constituents are *yeast mannan, yeast glucan* and *chitin*. Also fatty substances and probably proteins are present[2], the bulk being mannan and glucan.

Yeast mannan has been found by GARZULY-JANKE[6] to occur in nearly all species of yeast in a series examined by this author which included representatives of 12 genera. Mannan appeared to be absent only in *Nadsonia fulvescens*, *Rhodotorula glutinis* and *Schizosaccharomyces octosporus* and all *hyphal fungi* examined. The criterion for the presence of mannan was the formation of a copper mannan precipitate with Fehling solution in neutralised, cold 75% H_2SO_4 extracts of the organisms.

Yeast glucan is usually isolated by alkali digestion of the whole yeast. It is partly hydrolysed by boiling dilute mineral acids. The remaining part, which is extremely resistant to such reagents, then becomes soluble in alkali[7,4]. Recent details of its chemical structure and a review of earlier papers are given by BELL AND NORTHCOTE[8].

The study of pure cell walls[4] has revealed that the glucan residue after boiling the walls with 3% NaOH is *ca.* 25% of the original cell wall material. It dissolves up to *ca.* 50% in boiling 2% HCl. The remaining, now alkali-soluble, glucan (termed yeast hydro-glucan) appears as fibres in the electron microscope and yields a sharp X-ray diagram if the acid treatment is sufficiently prolonged. The same X-ray diagram is obtained if the alkali treatment is omitted and the cell walls are merely boiled with dilute acid. The hydro-glucan has been found to be identical with the reserve carbo-

References p. 9.

hydrate of the *Eugleninae*[9] known as paramylon, as far as may be concluded from the identical X-ray diagrams.

For the detection of yeast glucan no simple chemical method is available at present. Since the possibility of detection by X-ray diffraction mentioned above is a very recent development, the distribution of this substance among the yeasts has not yet been extensively investigated. In all yeast species examined by this method to date, 7 in all[4], the glucan has been found to be present.

Chitin has been detected micro-chemically by ROELOFSEN AND HOETTE[10] in all yeasts they studied (30 species distributed over 13 genera), except in *Schizosaccharomyces octosporus*. The presence of chitin was demonstrated by the formation of chitosan sulphate crystals with an improved van Wisselingh-Brunswik method. However, because of an observation mentioned later in this paper (see under *Phycomyces*), this method requires slight revision.

Somewhat earlier attempts to detect chitin in yeasts by X-ray diffraction had failed for most species[11]. Nevertheless our own experience is that the X-ray diagrams of acid-treated or alkali- and acid-treated cell walls of most species examined also exhibit faint, or even rather strong, lines of chitin, and always a complete chitin diagram was obtained if the hydro-glucan was dissolved by alkali, except with *Schizosaccharomyces*. This seems to confirm the statement of ROELOFSEN AND HOETTE. Probably the earlier failure was due to the method used for isolating the chitin, *viz.* repeated extraction of the whole yeast with 10% alkali according to SCHOLL[12], which seems to be less sensitive to small quantities than the method involving acid treatment indicated above and where pure cell wall material is used.

On account of the above observations yeast glucan and chitin are easily detected in yeasts, even simultaneously, by X-ray diffraction. Moreover, comparison of the hydro-glucan and chitin interferences in the X-ray diagram permits an estimation of the quantities of these substances in the specimen.

The above-mentioned data give rise to three queries which have formed the main subject of the work reported in the present paper; they may be summarised as follows:

1. Is yeast glucan a basic constituent characterising the cell walls of all yeasts, or do yeasts exist in which this polysaccharide is lacking, as is the case with mannan and chitin?

2. Is the occurrence of this substance restricted to the yeasts, as seems to be the case with the yeast mannan[6], or is it also present in the cell walls of other fungi?

3. In the cell walls of those fungi where the mannan is absent, is it replaced by constituents other than yeast glucan and chitin? In other words, are the three components the exclusive building stones of cell walls of fungi, apart from the cellulose detected in certain groups, fatty or waxy substances and proteins? And if not, will X-ray diffraction reveal other constituents?

In connection with (3) above, we could find no reference in the literature to the constitution of chemically intact pure cell walls of fungal tissues.

MATERIAL AND METHODS

Absence of glucan, we thought, might be expected most likely in the yeast species with cell walls of abnormal constitution in that mannan, or mannan and chitin, are absent. The cell walls of these yeasts were therefore studied first, together with some related and other species.

When it appeared that the method used for isolating yeast cell walls (vibration of a yeast

References p. 9.

suspension with small glass beads in a Mickle shaker and collecting the cell walls by centrifuging[1]) could also be applied under certain conditions (bigger size glass beads: *ca.* 250 μ against usual *ca.* 175 μ, young mycelium) to the mycelium of hyphal fungi, the research was extended to some of these, more or less arbitrarily chosen. It has to be recognised, however, that the methods of cleaning mycelial walls are not as yet quite satisfactory. In particular *Phycomyces* offered difficulties in this respect.

The yeasts were grown on malt agar plates, the fungi in malt-extract shaking cultures.

The treatment of the cell wall material consisted mainly in an extraction with warm (60° C) 3% NaOH for 30′ and (or) boiling 2% HCl for 1–2 hours. Afterwards the residues and the products that were recovered from the supernatant liquids by neutralising, alcohol precipitation or adding hot Fehling solution, were washed, dried and subsequently X-rayed.

The Fehling solution was added to the supernatant after extraction of the cell walls with warm 3% NaOH to ascertain the absence of mannan. A blue precipitate was regarded as copper-mannan, although other polysaccharides may also form a copper-complex[13].

Normally the quantities of cell wall material used for the experiments amounted from 50–100 mg, and the quantities of alkali or acid used were *ca.* 1 ml to each 10 mg cell wall material.

The X-ray diagrams were taken at a specimen-film distance of 40 mm with Cu$K\alpha$ radiation (Ni filtered) and glass pinholes 40 mm long by 0.5 mm diam. The reproduction is at natural size.

RESULTS

Yeasts

Schizosaccharomyces octosporus

In confirmation of previous data, mannan[6] and chitin[10] could not be detected, and in contrast with other yeasts the alkaline extract of the cell walls yielded a thick, flocculent precipitate when it was neutralised. The weight of the washed and dried precipitate was about 30% of that of the initial cell wall material. The X-ray diagram showed a set of rather sharp interferences (Fig. 1, I) different from that of hydro-glucan (Fig. 1, II). The weak, innermost reflection points to a slight contamination with yeast glucan as will appear later (see *Penicillium*).

The strongest interferences of the precipitate are also present in the X-ray diagram of the untreated cell walls and disappear after a sufficiently long boiling of the cell walls with dilute HCl, to give place to the hydro-glucan interferences. The hydro-glucan residue was about 10% of the initial cell wall material.

There are two further species recognised in the genus *Schizosaccharomyces, viz. S. pombe* and *S. versatilis*[14]. They are clearly differentiated from each other and from *S. octosporus* on morphological and physiological grounds. It seemed interesting therefore to ascertain whether the cell wall constitution is in harmony with the principles underlying the classification of these yeasts in one genus. Our results showed that in all three species the cell wall constitution is essentially the

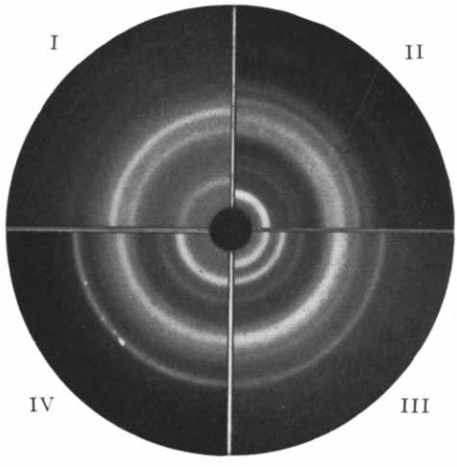

Fig. 1. Quadrants of X-ray powder diagrams of: I. precipitate formed in neutralised 3% NaOH extract (30′ at 60° C) of *Schizosaccharomyces octosporus* cell walls; II. *S. octosporus* cell walls after boiling for 2 hours with 2% HCl (hydro-glucan); III. *Nadsonia fulvescens* cell walls treated with 3% NaOH for 15′ at 100° C and boiling 2% HCl for 1 h (mixture of chitin and hydro-glucan); IV. *Sporobolomyces roseus* cell walls, same treatment as *Nadsonia* (chitin only).

References p. 9.

same. Thus taxonomic conclusions could be completely supported by an examination of the cell walls.

Nadsonia fulvescens

The absence of mannan in this species[6] was confirmed. Chitin (in much larger quantities than with baker's yeast and other mannan-containing species studied earlier[4]) and hydro-glucan were the main constituents of the residue after boiling with dilute acid or alkali and acid successively (Fig. 1, III). It is to be noted that the constitution of the alkali-soluble part of the cell walls (more than 60%) is unknown.

Rhodotorula glutinis

The absence of mannan was confirmed and the glucan was also found lacking in this species. In the residues after acid boiling or successive alkali and acid boiling the X-ray diagram demonstrated the presence of chitin as the sole microcrystalline product.

The genus *Rhodotorula* classified in the family *Cryptococcaceae* is characterised by the presence of a red to yellow carotenoid pigment. The same feature is found in the genus *Sporobolomyces*, belonging to the family *Sporobolomycetaceae*. It seemed interesting therefore to compare the cell walls of *Rhodotorula* with those of *Sporobolomyces*.

Sporobolomyces roseus

The investigation of the cell walls revealed that here again mannan and glucan are lacking. Only chitin could be detected (Fig. 1, IV), but this accounts for only *ca.* 10% of the whole cell wall in both this and the preceding yeast.

Questions may be raised as to the existence of a general correlation between the ability to produce carotenoid pigments in yeasts and the absence of yeast glucan; further by what components the mannan and glucan are replaced. This is being investigated.

Endomycopsis, Endomyces and Eremascus

These genera are regarded as transition forms between the yeasts and the moulds. Their cell wall constitution might be of interest therefore for a comparison with that of true yeasts on one hand and that of moulds on the other. The following species were studied: *Endomycopsis capsularis*, *Endomyces decipiens* and *Eremascus fertilis*. They all form true mycelium, and, in accordance with the rule of GARZULY-JANKE, nocop per mannan was obtained from the alkali extract of the two last species on adding Fehling solution. A small quantity of precipitate was obtained from the cell walls of *Endomycopsis*.

In all three species after treatment with dilute acid, the insoluble part of the cell wall was found to consist mainly of chitin and hydro-glucan. As with baker's yeast, this residue consists of 20–25% of the native wall, but the chitin content is much higher. In *Endomyces* chitin predominates over hydro-glucan, in the other two species the reverse is the case.

On neutralising the alkaline extract of *Endomyces* cell walls, a small amount of precipitate was obtained which gave a similar X-ray diagram to the precipitate from *Schizosaccharomyces* cell walls prepared in the same manner.

References p. 9.

OTHER FUNGI

Our study of the cell walls of moulds and the higher fungi has so far involved only three species. Nevertheless some results seem sufficiently interesting to be mentioned.

Penicillium notatum

In confirmation of GARZULY-JANKE's work with moulds, no mannan was obtained from the cell walls. However, by neutralising the alkaline extract, a considerable quantity of precipitate was obtained amounting to about 30% of the original cell wall material. The X-ray diagram (Fig. 2, I) revealed that the substance was the same as that obtained from *Schizosaccharomyces spp.* The weak innermost reflection in the diagram of the precipitate from *Schizosaccharomyces spp.*, corresponding to the strong innermost hydro-glucan interference, is not observed in the diagram of the precipitate from *Penicillium*. This is evidence that the former precipitate is contaminated by yeast glucan. According to the paper chromatogram of the hydrolysed substance, made in this laboratory by Mr P. KOOIMAN, it seems to be built up mainly of glucose residues. A further study is in progress.

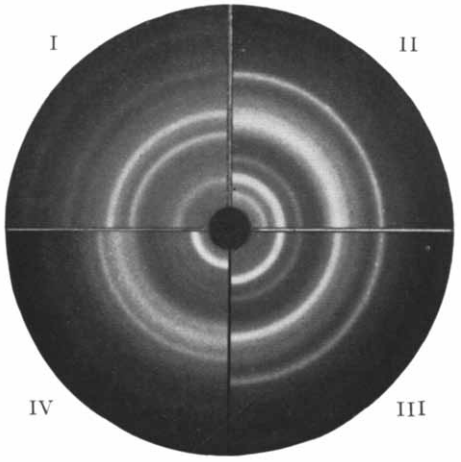

Fig. 2. Quadrants of X-ray powder diagrams of products from *Penicillium* cell walls: I. precipitate in neutralised 3% NaOH extract of the walls (30′ at 60° C); II. walls after above extraction and boiling with 2% HCl for 1 h (mixture of hydro-glucan and chitin); III. the same, but hydro-glucan dissolved with alkali (chitin); IV. same as II, but chitin dissolved with cold 30% HCl (hydro-glucan).

The residue, after boiling with dilute acid or treating with warm dilute alkali and boiling dilute acid successively, appeared to constitute about 30% of the original cell wall material and gave an X-ray diagram showing chitin and hydro-glucan interferences (Fig. 2, II). The presence of these substances was confirmed, as seemed desirable in particular for the hydro-glucan, by dissolving the hydro-glucan with alkali (Fig. 2, III) or the chitin with cold 30% HCl (Fig. 2, IV). The proportions of hydro-glucan and chitin in the residue were *ca.* $^1/_3$ and $^2/_3$ respectively.

It is remarkable that *Schizosaccharomyces* cell walls contain a substance which has not been found to date in budding yeasts, but which was detected in *Endomyces* and the very first mould studied along the new lines; also, as will appear later, it was isolated in small amounts from the fruiting body of *Agaricus campestris*. This seems to emphasise the close relationship between *Schizosaccharomyces* and the hyphal fungi, a relationship already indicated by its vegetative reproduction.

Phycomyces blakesleeanus

a. *Mycelium*

After boiling the cell wall material with 2% HCl for two hours the residue gave an X-ray diagram (Fig. 3, I) which did not resemble those usually obtained from acid-

References p. 9.

treated cell walls. Apart from three weak interferences corresponding to certain chitin lines the diagram showed a set of unknown reflections and no hydro-glucan interferences.

After subsequent extraction of the cell walls with 3% NaOH (30′ at 60° C) they give an X-ray diagram corresponding to that of chitin (Fig. 3, II). Only a trace of the most intense of the above unknown reflections is left. Apparently these reflections belong to an acid-insoluble component of the walls, which is readily soluble in warm dilute alkali.

In the X-ray diagram of the native wall there is no indication of the presence of the above component. Probably it is present in the native wall in an alkali-soluble complex, for if the walls are treated for 30′ with boiling 3% NaOH (60° C is insufficient) and subsequently with boiling 2% HCl, they yield a chitin diagram without the unknown interferences.

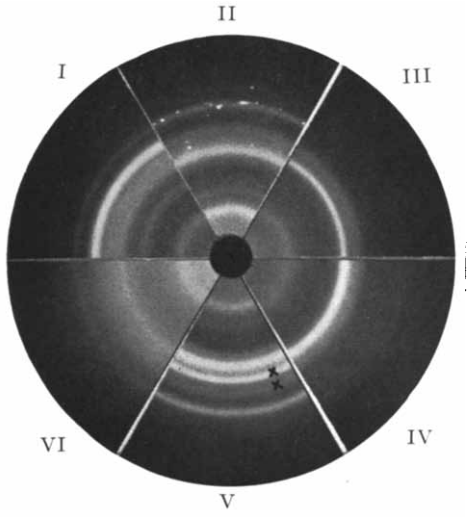

Fig. 3. Sectors of X-ray powder diagrams of *Phycomyces* cell walls and extracted products: I. walls after a 2 hours boiling with 2% HCl; II. after subsequent extraction with 3% NaOH (30′ at 60° C); III. precipitate in neutralised acid extract; IV. chitosan prepared from crustacean chitin; V. sporangiophore walls treated with 3% NaOH for 30′ at 60° C and 2% boiling HCl for 1 h (chitin and waxes (X)); VI. native sporangiophore walls.

The above complex seems to be soluble also in 2% HCl at 60–80° C. This may be inferred from the observation that after extraction with acid under these conditions and subsequent boiling of the clear supernatant, a brown flocculent precipitate develops which gives rise to the unknown X-ray interferences but not to chitin lines.

When the acid extract of the mycelium cell walls was neutralised, it was noted with interest that a precipitate was formed, in contrast with our experience with all other fungi examined so far. This precipitate represented about 10% of the original cell wall material and gave an X-ray diagram corresponding to that of chitosan, *i.e.* the deacetylation product obtainable from chitin after heating in concentrated alkali. In Fig. 3, III, the diagram of the substance is shown matched against that of a sample of chitosan prepared from crustacean chitin (Fig. 3, IV). As a confirmation that we are dealing with chitosan, the precipitate formed the characteristic chitosan sulphate spherocrystals when treated with H_2SO_4 according to published methods of preparation[15]. The spherocrystals were negatively birefringent and indistinguishable from the chitosan sulphate crystals prepared from the alkali-treated crustacean chitin. The slightly higher definition of the X-ray diagram of the fungal chitosan and the bigger size of the largest spherocrystals it produced, as compared with those from the control substance, suggest that it is in an even more nearly pure state than the latter. Since chitosan is readily soluble in dilute acids but not in neutral solvents[15], it is understandable that it precipitated from the acid extract of the cell walls on neutralisation.

As far as we know, this is the first observation of the occurrence of chitosan in plant cell walls and probably in living nature. However, the "lycoperdine" studied by KOTAKE AND SERA[16] might be identical with chitosan. We hope to be able to go into this problem at a later date.

References p. 9.

It is to be noted that chitin is usually detected microchemically by first transforming it into chitosan and then identifying this substance by methods indicated above. As a rule, no precautions are taken to see that chitosan, possibly present in the original material, is first removed. Therefore, in most cases where chitin is said to be detected microchemically, confusion with chitosan is possible, unless ancillary X-ray evidence is available. It is clear that, in the chitosan sulphate test for the detection of chitin, acid extraction should precede the conversion of chitin into chitosan to make the method unambiguous.

b. *Sporangiophores*

The sporangiophores of *Phycomyces* have been the subject of extensive investigations concerning spiral growth, tropism and cell wall structure. As to the chemical constitution we know only that chitin is a major constituent. It seemed interesting to examine the sporangiophore walls for comparative purposes along similar lines to the mycelial walls.

In sporangiophore cell walls from a culture on bread, large differences from the mycelial cell walls could be observed. In contrast to the acid-treated mycelial walls the acid-, or alkali- and acid-treated, sporangiophore walls gave an X-ray diagram (Fig. 3, V) showing clear chitin lines. Furthermore, the unknown reflections in the diagrams of the former walls were absent in those of the latter, and strong lines (marked X in Fig. 3, V) indicated the presence of a high amount of waxy or fatty substances. From weighings and chloroform extraction it was concluded that *ca.* 55% of the walls consisted of roughly equal amounts of chitin and waxy or fatty substances. Chitosan was extracted with dilute HCl, but it would not crystallise sufficiently for identification by X-ray diffraction. It was identified by the chitosan sulphate test. The chitosan sulphate spherulites had a yellow colour, presumably due to an impurity which may also have prevented the crystallisation of the chitosan.

A substance was also obtained which showed in the diagram the 2nd–5th order reflections of a long crystal spacing of 60.5 A. It was formed on neutralisation of an extract with boiling 2% HCl from the residue of the cell walls after treatment with 3% NaOH. This substance may be identical with that reported by CASTLE[17] to be dissolved from the alkali-treated sporangiophore walls in dilute acid and to affect strongly the optical behaviour of the walls.

It is to be noted that neither the fatty substances of the sporangiophore walls nor the acid-insoluble component of the mycelium cell walls revealed themselves in the X-ray diagrams of the native walls.

The native walls in both cases give somewhat vague rings (Fig. 3, VI) corresponding to the most intense interferences of chitin; these are slightly more distinct in the sporangiophore diagram. Apparently the constituents of the native cell wall are partly prevented from forming crystallites, presumably on account of linkages by which they are united to form one or more complex compounds.

Agaricus campestris

The cell wall material examined here was from the stem of a young fruit body. It yielded the highest amount of chitin we have obtained so far from fungal cell walls, *viz.* 30–35%; in addition, a small amount of the "*Schizosaccharomyces* polysaccharide" was precipitated on neutralising the alkali extract of the cell walls. The occurrence of

References p. 9.

the latter substance in this species and in *Penicillium sp.* suggests that it might be widespread among fungi except in budding yeasts.

The present observations may have shown the value of X-ray diffraction and the necessity of starting from mechanically isolated cell wall material in the study of the cell wall constitution of fungi. It seems likely that these methods combined with paper chromatography and other modern chemical methods may lead to a much more complete conception of the constitution of fungal cell walls than has hitherto been possible.

CONCLUSIONS

1. The poly-glucosan known as yeast glucan is lacking in the pink yeasts *Rhodotorula glutinosa* and *Sporobolomyces roseus*. Yeast mannan is also lacking, as had been noted before for *Rhodotorula*.

2. Yeast glucan is present in other mannan-deficient yeast species, *e.g. Nadsonia fulvescens* and *Schizosaccharomyces octosporus*, and in certain species which are regarded as transition forms between the yeasts and the moulds.

3. All mannan-deficient species mentioned above (except *S. octosporus*) have an increased chitin content, but the amount is smaller than is normally the amount of mannan in other species. We cannot say, therefore, that in the cell walls of the mannan-deficient species mannan is replaced by chitin. It is mainly replaced by other, hitherto unknown, substances.

4. In all three species recognised at present in the genus *Schizosaccharomyces* both mannan and chitin appear to be absent. An unknown alkali-soluble microcrystalline component consisting of about 30–35% of the cell wall occurs in these species in addition to yeast glucan; this is probably a different glucan.

5. The cell walls of *Penicillium notatum* contain about 30% of essentially the same unknown substance as just mentioned. In addition yeast hydro-glucan (*ca.* 10%) and chitin (*ca.* 20%) were obtained from the walls of this species.

6. A small amount of the above unknown component occurs in the cell walls of *Endomyces decipiens* and the stem of the fruit body of *Agaricus campestris*. The latter contains no yeast glucan and a high amount of chitin (*ca.* 35%).

7. In the mycelial walls of *Phycomyces blakesleeanus*, after boiling with 2% HCl, a rather small amount of chitin is present in addition to an unknown microcrystalline component which is soluble in warm 3% NaOH. Furthermore, these cell walls appear to contain chitosan. Yeast glucan is absent.

8. The sporangiophore walls of *Phycomyces* have a different constitution. They contain roughly 25% chitin and nearly as much waxy or fatty substances. An acid-soluble component with a long crystal spacing of 60.5 A was obtained from the alkali-treated walls. Chitosan is also present.

9. The usual microchemical method of chitin detection needs to be revised in that a treatment with acid should precede the conversion of chitin into chitosan.

10. The X-ray evidence suggests that as a rule in the native cell walls of fungi the constituents are linked by more or less easily hydrolysable bonds to form complexes.

References p. 9.

ACKNOWLEDGEMENTS

The author is indebted to Dr M. P. SCARR, Hayes, for corrections of the English text and to Miss M. F. DE REGT for valuable assistance in the work. The yeast strains were supplied by the Yeast Division of the Centraal Bureau voor Schimmelcultures, Delft. The work has been supported by the Netherlands Organisation for Pure Research (Z.W.O.).

SUMMARY

The occurrence of known cell wall constituents of yeasts, *viz.* yeast mannan, yeast glucan and chitin, in certain yeasts and some other fungi has been investigated using pure, chemically intact cell wall material and applying X-ray diffraction for the detection of chitin and yeast glucan.

Considerable differences between the cell walls of certain yeasts and between those of certain other fungi have been established.

Several new fungal cell wall components with characteristic X-ray diagrams have been detected.

RÉSUMÉ

La présence de constituants connus des parois cellulaires de levures (mannane de levure, glucane de levure et chitine) chez certaines levures et quelques autres champignons a été étudiée sur des parois pures et chimiquement intactes, en utilisant la diffraction des rayons X pour la détection de la chitine et de la glucane. Des différences importantes entre les parois cellulaires de certaines levures et entre celles de divers autres champignons ont été établies.

Plusieurs nouveaux constituants des parois cellulaires, possédant des diagrammes de rayons X caractéristiques, ont été mis en évidence.

ZUSAMMENFASSUNG

Das Vorkommen von bekannten Zellwandbestandteilen der Hefe, nämlich Hefemannan, Hefeglucan und Chitin in gewissen Hefen und einigen anderen Pilzen wurde, unter Verwendung von Röntgendiagrammen zur Auffindung von Chitin und Hefeglucan, an reinem, chemisch intakten Zellwandmaterial untersucht. Es wurden beträchtliche Unterschiede zwischen den Zellwänden bestimmter Hefen und denen gewisser anderer Pilze festgestellt.

Mehrere neue Pilzzellwandbestandteile mit charakteristischen Röntgendiagrammen wurden gefunden.

REFERENCES

1 H. MICKLE, *J. Roy. Microscop. Soc.*, 68 (1948) 10.
2 D. H. NORTHCOTE AND R. W. HORNE, *Biochem. J.*, 51 (1952) 232.
3 A. L. HOUWINK, D. R. KREGER AND P. A. ROELOFSEN, *Nature*, 168 (1951) 693.
4 A. L. HOUWINK AND D. R. KREGER, *Antonie van Leeuwenhoek*, 19 (1953) 1.
5 P. A. ROELOFSEN, *Biochim. Biophys. Acta*, 10 (1953) 477.
6 R. GARZULY-JANKE, *Zentr. Bakt. Parasitenk.*, 102 (1940) 361.
7 R. A. MCANALLY AND I. SMEDLEY-MACLEAN, *Biochem. J.*, 31 (1937) 72.
8 D. J. BELL AND D. H. NORTHCOTE, *J. Chem. Soc.*, (1950) 1944.
9 D. R. KREGER AND B. J. D. MEEUSE, *Biochim. Biophys. Acta*, 9 (1952) 699.
10 P. A. ROELOFSEN AND ILSE HOETTE, *Antonie van Leeuwenhoek*, 17 (1951) 297.
11 R. FREY, *Ber. Schweiz. Botan. Ges.*, 60 (1950) 199.
12 E. SCHOLL, *Monatsh. Chem.*, 29 (1908) 1023.
13 H. DEUEL AND H. NEUKOM, *Makromolekulare Chemie*, 4 (1949) 91.
14 J. LODDER AND N. J. W. KREGER-VAN RIJ, *The Yeasts*, Amsterdam (1952).
15 H. BRUNSWIK, *Biochem. Z.*, 113 (1921) 111.
16 Y. KOTAKE AND Y. SERA, *Z. physiol. Chem.*, 88 (1913) 56.
17 S. E. CASTLE, *Protoplasma*, 31 (1938) 331.

Received June 22nd, 1953