Cell wall degradation in the autolysis of filamentous fungi

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Abstract

A systematic study on autolysis of the cell walls of fungi has been made on *Neurospora crassa, Botrytis cinerea, Polystictus versicolor, Aspergillus nidulans, Schizophyllum commune, Aspergillus niger,* and *Mucor mucedo.* During autolysis each fungus produces the necessary lytic enzymes for its autodegradation. From autolyzed cultures of each fungus enzymatic precipitates were obtained. The degree of lysis of the cell walls, obtained from non-autolyzed mycelia, was studied by incubating these cell walls with and without a supply of their own lytic enzymes. The degree of lysis increased with the incubation time and generally was higher with a supply of lytic enzymes.

Cell walls from mycelia of different ages were obtained. A higher degree of lysis was always found, in young cell walls than in older cell walls, when exogenous lytic enzymes were present.

In all the fungi studied, there is lysis of the cell walls during autolysis. This is confirmed by the change of the cell wall structure as well as by the degree of lysis reached by the cell wall and the release of substances, principally glucose and N-acetylglucosamine in the medium.

Introduction

Autolysis is a natural degradation process which starts after exhaustion of the external carbon supply and reserve substances. The degradation process take place in the cytoplasm (4) as well as in the cell wall (20). Cell wall lysis is carried out by lytic enzymes which may be located on the wall (14, 16) or excreted into the medium during the autolytic phase of growth (9, 19, 21). The breakdown products from the cell wall may be reutilized for the synthesis of wall polysaccharides in the cell wall turnover (30). During autolysis, it is confirmed that the synthesis of lytic enzymes implicated in cell wall degradation, occurs (22). The synthesis is probably induced by substances released from the degraded cell walls, being regulated by the living cells, the low level of inducers required (3, 17).

In the present investigation we describe the lysis of walls of the fungi *Neurospora crassa, Botrytis cinerea, Polystictus versicolor, Aspergillus nidulans, Schizophyllum commune, Aspergillus niger,* and *Mucor mucedo* of different ages by endogenous lytic enzymes and by a supply of their own lytic enzymes produced during autolysis of each fungus.

Materials and methods

Organisms and culture conditions

Nerospora crassa 2 254 CECT (Colección Española de Cultivos Tipo). *Botrytis cinerea* Pers. ex. Fr. was obtained from Dr. J. A. Leal (Instituto de Inmunología y Biología Microbiana C.S.I.C., Spain) who isolated the pathogen and had its identity confirmed by the Centraalbureau voor Schimmelcultures, Baarn (Netherlands), *Polystictus ver-*

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sicolor 255.10 CBS, *Aspergillus nidulans* 2 544 CECT, *Schizophyllum commune* 2 650 CECT, *Aspergillus niger* 120.49 CBS, *Mucor mucedo* 109.16 CBS.

The fungi were grown at 25 °C in stationary culture, in 1 000 mL conical flasks containing 200 mL of a mineral medium as follows (g/L): glucose (anhydrous), 10; ammonium tartrate, 2; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; yeast extract, 1.0; and trace element solution, 1 mL. The trace element solution contained the following (mg/L): $Na_2B_4O_7 \cdot 10H_2O$, 100; $ZnSO_4 \cdot 7H_2O$, 70; $FeSO_4 \cdot 7H_2O$, 50; $CuSO_4 \cdot 5H_2O$, 10; $MnSO_4 \cdot 4H_2O$, 10; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10. The flasks and their contents were plugged with non absorbent cotton wool and sterilized by steaming for 20 min on each of 3 succesive days. Each flask was inoculated with 1 mL of a spore suspension or mycelial suspension obtained by adding 5 mL sterile distilled water to malt agar slopes of each fungus.

Production of autolytic enzymes

Samples were taken after the cultures had reached the end of the autolysis to precipitate the lytic enzymes in the culture fluid. Microscopic checks on sterility were carried out for every sample. The culture fluid was separated from the mycelium by centrifugation and the lytic enzymes precipitated using the tannic acid method (24). The dry precipitates were stored at 4 °C and the activities of the lytic enzymes, which were stable for at least 9 months, were tested periodically.

Wall preparation

Cell walls from each fungus at different ages, were obtained by freezing the mycelium at -20 °C, grinding with a pestle and mortar, breaking in the ultrasonic desintegrator (MSE model MKZ, 150 W), and washing by repeated centrifugation with 0.1 M NaCl, 0.5 M acetate buffer pH 5.5 and distilled water until the cell walls were free from cytoplasmic materials as seen in the light microscope.

Enzyme assays

The enzymes 1,3-β-glucanase (exoglucanase, E.C. 3.2.1.6), invertase (E.C. 3.2.1.26), β-N-acetylglucosaminidase (E.C. 3.2.1.30), chitinase (E.C. 3.2.1.14), acid phosphatase (E.C. 3.1.3.2), and alkaline phosphatase (E.C. 3.1.3.1) were assayed as described by Lahoz *et al.* (1976); polygalacturonase (E.C. 3.2.1.15) was assayed by the method of Archer and Fielding (1975). The enzymes 1,3-(4)-β-glucanase (endoglucanase, E.C. 3.2.1.6), amylase (E.C. 3.2.1.1), 1,6-β-glucanase (E.C. 3.2.1.11), dextranase, (E.C. 3.2.1.54), and 1,3-α-glucanase (E.C. 3.2.1.84) were assayed by the hydrolysis of the substrates detailed below at final concentration of 1 mg/mL in 0.05 M borate citrate-phosphate buffer pH 5.5 at 37 °C. The enzyme 1,3-(4)-β-glucanase was assayed using 1,3-(4)-β-glucan (Lichenan, Koch Light Laboratories LTD England), amylase using soluble starch (E. Merck, Germany), 1,6-β-glucanase using the 1,6-β-glucan (pustulan) obtained by the method of Lindberg and McPherson (1962) (11), cellulase using carboxymethylcellulose sodium salt (Serva), dextranase using dextran T_{10} (Pharmacia Fine Chemicals) and 1,3-α-glucanase using 1,3-α-glucan obtained from *Aspergillus nidulans* at described by Zonneveld (1971) (31). The reducing sugars released by these enzymes were measured by the Somogyi-Nelson method (25, 15). One unit of enzyme was defined as that amount which released 1.0 μmol of product per minute at 37 °C.

Determination of enzyme activities in walls 'in situ'

Walls from cultures of different ages were suspended at 0.1% (w/v) in 0.05 M borate-citrate-phosphate buffer pH 5.5 and the suspensions were assayed for the different enzymes studied. After stopping the reaction, the walls were separated by centrifugation and the enzyme product appearing in the supernatant was measured.

Wall degradation

This was measured by a method based on that of Hughes (1971) (7). A cell wall suspension (1 mg/mL) in 0.05 M borate-citrate-phosphate buffer, pH 5.5, was subjected to ultrasonic treatment for 1 min as described earlier. An enzyme preparation in the same buffer, obtained by dissolving precipitated culture filtrate (see above) at 1 mg/mL was filtered through a Millipore filter (pore size 0.22 μm). One mL of the cell wall suspension was incubated with

1 mL of the enzyme solution at 37 °C in presence of 0.5 mM sodium azide. The decrease in E_{675} during the incubation period was taken as a measure of cell wall lysis. Bacterial contamination was not detected. The release of substances to the medium from the cell wall degradation was determined as follow, free glucose as described by Santamaría *et al.* (1978), reducing substances by the method of Somogyi (1945) in conjunction with that of Nelson (1944) using glucose as standard, total carbohydrates by the anthrone method of Trevelyan and Harrison (1952) using glucose as standard and N-acetylglucosamine by the method of Tracey (1955).

Electron microscopy

Cell walls for shadow casting were coated with gold/palladium (60:40) at an angle of 15°.

Results

Under the described conditions, the degree of autolysis (percentage loss in mycelium dry weight) (20) reached the following values, 80.3, 80.0, 76.0, 75.0, 60.0, 50.0, and 50.0 after sixty days of incubation for *N. crassa, B. cinerea, P. versicolor, A. nidulans, S. commune, A. niger,* and *M. mucedo* respectively. The pHs of these autolyzed cultures were 6.6, 6.7, 6.2, 7.0, 6.5, 6.7, and 4.8 respectively.

Similarly the proteins precipitated from the autolyzed cultures were 97.3, 55.5, 72.0, 72.7, 30.4, 66.0 and 30.0 mg respectively for each sample (200 mL of filtrate culture fluid). Table 1 shows the enzymatic activities found in the precipitates from autolyzed cultures. Some lytic enzymes found in the precipitates of each fungus were also present in wall preparations from each fungus, as can be seen in Table 2.

Figure 1 shows the degree of lysis of the cell walls of each fungus by its own lytic enzymes, with and without the addition of an exogenous supply of lytic enzymes. The increase of this degradation with the incubation time occurs in all the cell walls of different ages studied, but the representation was made in cell walls from cultures not autolyzed (3 or 4 days old, see method). This degradation occurs to a lesser extent in walls incubated without the addition of an exogenous supply of lytic enzymes from cultures autolyzed, Figure 1 also shows the gradual decrease in the degree of lysis after 120 h of incubation of the cell walls from the different fungi and from cultures of different ages (for the same fungus), with and without the addition of an exogenous supply of lytic enzymes.

Table 3 shows the concentrations of total carbohydrates, reducing substances, glucose and N-acetylglucosamine released from cell walls of different ages into the medium after 120 hours of incubation in those cultures with a supply of exogenous lytic enzymes.

Plate 1 shows the shadowed appearance of the cell wall of each fungus (from cultures of three or

Table 1. Enzymatic activities determined in the protein precipitates obtained from autolyzed cultures of different fungi.

Enzymes (mU/mg protein)	Fungi						
	N. crassa	*B. cinerea*	*P. versicolor*	*A. nidulans*	*S. commune*	*A. niger*	*M. mucedo*
Chitinase	63.9	16.1	27.2	386.5	0.0	2.1	0.0
β-N-acetylglucosaminidase	99.3	117.3	86.9	700.0	16.9	32.4	1.9
Acid phosphatase	424.7	0.0	27.7	12.4	23.4	0.0	0.6
Alkaline phosphatase	47.9	0.0	0.8	9.2	0.0	10.0	0.0
1,3-β-glucanase	2 232.3	583.3	1 157.2	348.3	1 241.2	1 739.4	400.0
Invertase	2 848.9	0.0	1 657.2	412.7	159.7	0.0	0.0
Cellulase	94.5	0.0	166.6	24.8	162.7	40.3	0.0
Dextranase	123.3	0.0	4.4	0.0	0.0	0.0	60.0
Polygalacturonidase	132.8	5.3	250.0	0.0	13.7	222.1	0.0
1,3-(4)-β-glucanase	219.1	34.2	194.4	0.0	254.7	0.0	0.0
1,6-β-glucanase	46.4	43.8	92.5	0.0	50.8	13.0	215.3
Amylase	1 726.6	425.9	407.2	24.8	691.9	0.0	1 940.0
1,3-α-glucanase	0.0	0.0	0.0	0.2	0.1	0.0	0.0

Results are the mean of three replicates

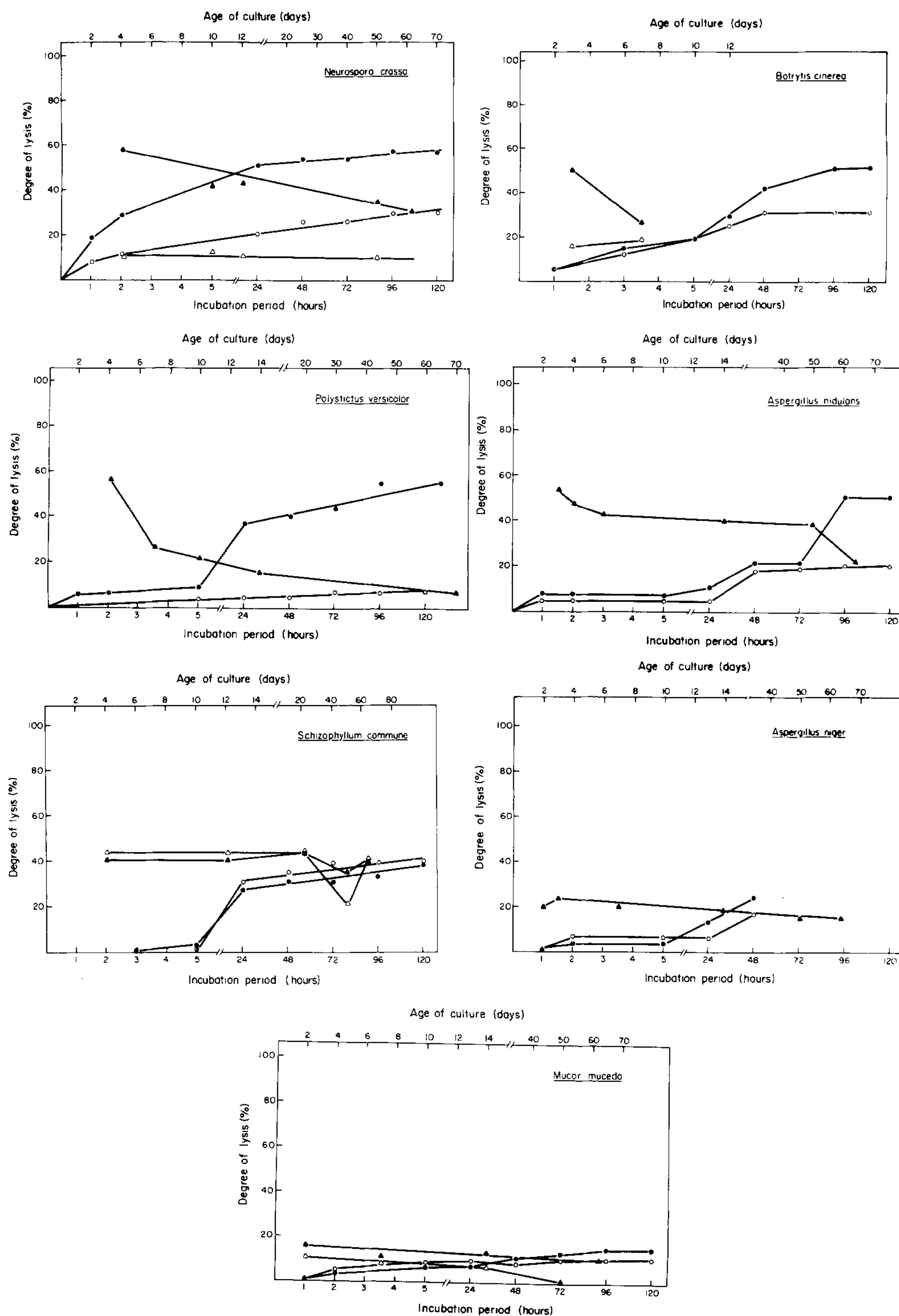

Fig. 1. Degree of lysis reached by cell walls of different fungi obtained from non-autolyzed mycelia with (●) and without (○) the addition of a supply of their own lytic enzymes, obtained from autolyzed cultures of each fungus after varying the incubation period, and degree of lysis reached by cell walls of different fungi obtained from mycelia of different ages, similarly with (▲) and without (△) the addition of lytic enzymes.

Table 2. Enzymatic activities determined 'in situ' in the cell walls obtained from non-autolyzed mycelia of different fungi.

Enzymes (mU/mg cell walls)	Fungi						
	N. crassa	*B. cinerea*	*P. versicolor*	*A. nidulans*	*S. commune*	*A. niger*	*M. mucedo*
Chitinase	0.0	6.8	0.0	0.0	0.0	0.0	0.0
β-N-acetylglucosaminidase	1.0	4.4	0.8	0.6	0.0	2.0	1.0
Acid phosphatase	2.6	0.0	2.0	3.4	1.0	23.0	1.7
Alkaline phosphatase	0.0	0.0	–	0.0	0.0	0.0	0.0
1,3-β-glucanase	13.2	3.1	8.2	8.2	4.0	12.6	8.2
Invertase	17.2	0.0	6.8	8.0	3.2	9.0	7.0
Cellulase	8.0	0.0	6.8	5.6	2.0	7.0	5.8
Dextranase	10.2	0.0	9.0	15.0	12.6	13.8	11.4
Polygalacturonidase	38.2	0.0	41.6	40.6	36.6	38.2	29.2
1,3-(4)-β-glucanase	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1,6-β-glucanase	0.0	2.5	0.0	0.0	0.0	0.0	11.4
Amylase	12.6	1.5	10.2	18.2	6.8	20.0	10.4
1,3-α-glucanase	0.0	0.0	0.0	1.3	0.1	0.0	0.0

Results are the mean of three replicates

Table 3. Release of total carbohydrates, reducing substances, glucose and N-acetylglucosamine to the medium, when cell walls of different ages from different fungi are treated with their own lytic enzymes obtained from autolyzed cultures.

Fungi	Cell walls (d)	Tot. carbo.[a]	Red. subst.[a]	Glucose.[a]	N-acetylglu.[b]
N. crassa	4	450.0	191.2	118.5	37.6
	12	413.0	164.7	92.3	24.5
	49	375.0	143.5	110.3	20.8
	63	225.0	23.9	10.1	0.0
B. cinerea	3	122.4	73.8	45.0	0.0
	7	433.6	286.2	144.0	64.5
P. versicilor	4	120.0	115.2	110.3	9.5
	7	–	–	68.4	0.0
	10	105.0	104.0	68.4	0.0
	14	95.0	90.0	80.0	3.8
	63	76.0	71.2	68.4	3.8
A. nidulans	3	152.0	116.8	49.5	67.6
	4	323.0	116.8	78.8	43.3
	14	226.0	175.3	45.0	67.6
	49	–	63.7	25.9	37.6
	63	–	103.5	42.8	48.8
S. commune	7	101.3	71.0	40.5	0.0
	12	142.5	100.0	45.0	0.0
	21	120.0	87.5	45.5	0.0
	49	105.0	62.5	42.3	0.0
	63	–	50.0	42.3	0.0
A. niger	2	247.0	181.5	179.2	10.7
	3	247.0	148.5	140.4	8.3
	14	228.1	121.0	115.2	8.8
M. mucedo	4	–	10.6	4.5	0.0
	7	45.0	16.0	4.5	0.0
	14	41.3	18.7	6.8	0.0
	49	63.8	13.3	4.5	0.0
	63	–	13.3	4.5	0.0

[a] μg of glucose/mg cell walls
[b] μg of N-acetylglucosamine/mg cell walls
Results are the mean of three replicates

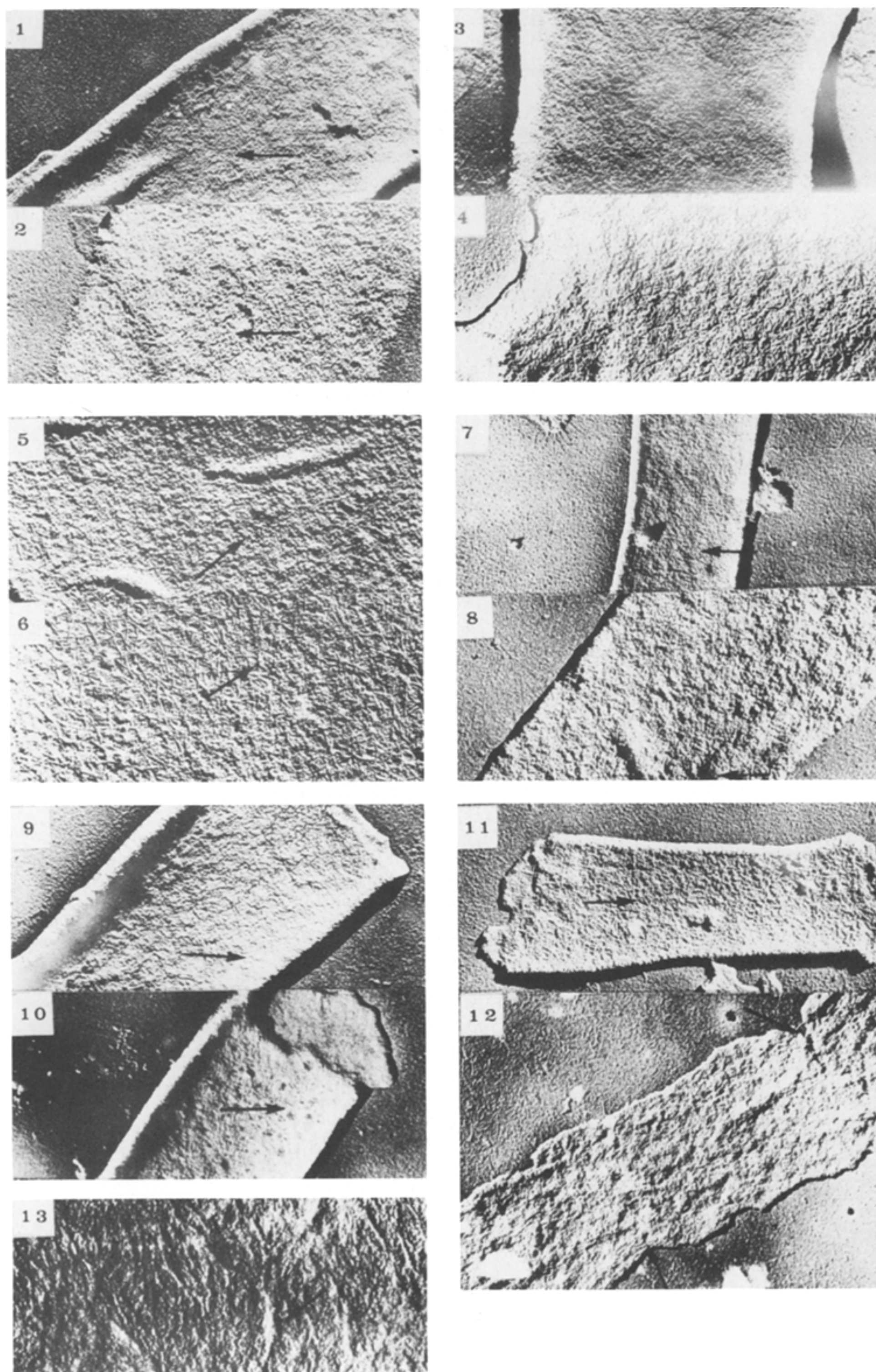

Plate 1. Electron micrograph of shadowed preparation of cell walls of different fungi showing appearance of controls (1, 3, 5, 7, 9, and 11) and appearance after treatment for 24 hours with their own lytic enzymes obtained from autolyzed cultures of each fungus (2, 4, 6, 8, 10, 12, and 13). *N. crassa* (1, ×15 and 2, ×15). *B. cinerea* (3, ×15 and 4, ×16). *P. versicolor* (5 ×18 and 6, ×19). *A. nidulans* (7, ×14 and 8, ×17). *S. commune* (9, ×16 and 10, ×13). *A. niger* (11, ×15 and 12, ×16). *M. mucedo* (13, ×19).

four days) before and after being treated for 24 h with a supply of exogenous lytic enzymes from each fungus.

Discussion

A systematic study on autolysis has been made on different strains of filamentous fungi. In each case a high degree of autolysis is obtained only when degradation of the cytoplasm and wall occurs. The natural autolysis of these fungi takes place at values of pH between 6.2 to 7.0 except for *M. mucedo* which occurs at pH 4.8. Generally the lytic enzymes which attack the cell walls and the reserve carbohydrates actuate at these pH values (19, 21, 5).

The enzyme, 1,3-β-glucanase is the only enzyme which is present at high levels in all the fungi studied, in the autolyzed cultures as well as 'in situ' in the cell walls. The maximum specific activity of 1,3-β-glucanase is presented in the enzymatic precipitated of *Neurospora crassa* with 2.2 U/mg of protein (see Tables 1 and 2), and the minimum activity is presented by *Mucor mucedo* with 0.4 U/mg of protein. A relationship can also be seen between the activity of 1,3-β-glucanase, the degree of autolysis and cell wall composition (2) of these strains.

In spite of the fact that chitin is another important component of the cell walls of these fungi, we have not found high activities of chitinase and β-N-acetylglucosaminidase in these autolyzed cultures with the exception of *Aspergillus nidulans* (see Table 1).

Amylase activity was present in all the enzymatic precipitates from these fungi, but not in *Aspergillus niger*. In these conditions *Aspergillus niger* does not produce amylase activity, although this fungus has been described as an industrial producer of this enzyme (6). In all the fungi studied, this enzyme was detected 'in situ' in the cell wall.

Similarly the degree of lysis of the cell walls of each fungus (Fig. 1) with the endogenous enzymes was smaller than when exogenous lytic enzymes were added, with exception of *Schizophyllum commune* and *Mucor mucedo*. These two fungi degraded the cell walls to a comparable extent in both cases.

The different degree of lysis found in walls from young and old cultures, when exogenous lytic enzymes were present, could be due to the disappearance, during autolysis, of the fractions of the cell walls most susceptible to digestion (20). Table 3 confirms these results; in the lysis of the cell wall, the total carbohydrates, reducing substances, glucose and N-acetylglucosamine released to the medium, always was bigger from young than from old cell wall. In the case of *Botrytis cinerea* and *Aspergillus niger,* under the described conditions older cell walls cannot be obtained.

The degree of lysis of different aged cell walls with endogenous lytic enzymes is not significant, because the older or more disrupted cell walls do not retain protein.

The behaviour of each fungus to the action of its own lytic enzymes is different as can be seen by the action of these enzymes on the cell walls in shadowed preparations of young cell walls of these fungi, before and after being treated with their own lytic enzymes during 24 hours. The structure of the cell walls of hyphae of *Neurospora crassa* showed a similar feature to that obtained by Hunsley and Burnett (1970) after treatment with laminarinase/pronase. Although chitin microfibrils can be seen, it is probable that some part of chitin microfibrils had been attached as the presence of N-acetylglucosamine in the medium confirms. The structure and composition of the cell wall of *N. crassa* (13, 12) is once more confirmed by the autolysis of the cell wall (Plate 1, 1 and 2). In *B. cinerea* and *P. versicolor* Plate 1, 3 and 4; 5 and 6) it is clear that the attachment of the cell wall of these fungi takes place in a similar manner to that in *N. crassa*. The microfibrils of chitin in both cases are more clearly defined having been removed from the amorphous mass of glucan. It is probable that in the case of *P. versicolor* an additional lysis of the protein of the cell wall can occur, because in the same conditions, this fungus produces during autolysis different proteolytic enzymes (18).

Untreated cell wall fragments of *Aspergillus nidulans* (Plate 1, 7) contain oriented fibrillar structures and an amorphous material. This structure is very similar to that observed by Troy and Koffler in *P. chrysogenum* (1969). After being treated by their own lytic enzymes for 24 h, the cell wall structures resemble each other closely to the untreated cell walls (Plate 1, 8) simultaneous are more porous. It is confirmed by the same release of glucose and

N-acetylglucosamine to the medium.

The structures of untreated and treated cell walls of *A. niger* (Plate 1, 11 and 12) can be compared with the cell walls of *A. nidulans* (Plate 1, 7 and 8) although in the medium the release of glucose was greater than N-acetylglucosamine, being in the ratio of 17 to 1.

In the outermost layer of S-glucan, the 1,3-α-glucan (8, 29) of the cell wall of *Schizophyllum commune* can be seen to have attacked points of this cell wall (Plate 1, 9 and 10) after 24 hours of hydrolysis with its own lytic enzymes. In this enzymatic complex is present 1,3-α-glucanase, which can attack this layer of S-glucan.

The gel-forming polysaccharide found in the cell walls of *Mucor mucedo* blocks the shadow casting preparation of young cell walls. After being treated with its own lytic enzymes a granulated and microfibrillar aspect can be seen.

In all the fungi studied it is confirmed that during autolysis there is lysis of the cell walls. This is shown by the change in the cell wall structures as well as by the degree of lysis reached for cell walls treated with their own lytic enzymes and the release of substances in the medium.

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