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Influence of Manganese on Morphology and Cell Wall Composition of *Aspergillus niger* During Citric Acid Fermentation*

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Abstract. Morphology and cell wall composition of *Aspergillus niger* were studied under conditions of manganese sufficient or deficient cultivation in an otherwise citric acid producing medium. Omission of Mn^{2+} (less than 10^{-7} M) from the nutrient medium of *Aspergillus niger* results in abnormal morphological development which is characterized by increased spore swelling, and squat, bulbeous hyphae. Fractionation and analysis of manganese deficient cell walls revealed increased chitin and reduced β-glucan contents as well as reduction of galactose containing polymers, as compared to cell walls from manganese sufficient grown hyphae. Addition of copper induced the same effect as manganese deficiency, both on morphology and cell wall composition. Addition of cycloheximide also produced a very similar type of morphology with increased chitin and reduced β-glucan contents of the cell wall but its effect on galactose was less pronounced.

Key words: Morphology – Manganese deficiency – Cell wall – *Aspergillus niger* – Citric acid fermentation

One of the scarcely studied features in citric acid fermentation by *Aspergillus niger* is the morphology of the fungus. It is well known from patent literature that only hyphae which form hard, compact pellets, produce citric acid. Clark et al. (1966) showed that manganese deficiency was the key factor in the expression of this type of morphology.

Abnormal morphology or development due to manganese deficiency has been reported for several fungi (Detroy and Ciegler, 1971; Barnett and Lilly, 1966; Tinell et al., 1973; Garrison and Boyd, 1974; Reiss and Nickerson, 1974). Zonneveld (1975) reported that inhibition of fruiting body formation under manganese deficient conditions was due to a lack of α-glucan synthesis. Mahadevan and Tatum (1965) have shown that changes in morphology of *Neurospora crassa* are correlated with changes in cell wall composition. It was thus of interest to investigate whether the 'citric acid producing' type of morphology of *Aspergillus niger* might be correlated with the contents of one or more polymer components of the cell wall.

Some aspects of the role of manganese ions in *Aspergillus niger* metabolism have recently been presented (Kubicek and Röhr, 1977, 1978; Kubicek et al., 1979; Orthofer et al., 1979). Influence on protein synthesis was considered to be of major importance, since cycloheximide was found to be able to antagonize the effect of manganese addition. Thus interest was focussed towards the question whether this could also account for the alterations in morphology.

Materials and Methods

Strain and Culture Conditions. Aspergillus niger B60 was used throughout these studies and was selected from *Aspergillus niger ATCC 11414* (Clark et al., 1966) by means of a paper culture technique (Röhr et al., 1979). The strain was kept on potato dextrose agar slants and subcultured every month. The composition of the medium and conditions for growing *Aspergillus niger* under citric producing pilot plant conditions have been reported previously (Kubicek and Röhr, 1978). Sucrose was passed through a cation exchange resin (Dowex AG 50W × 8) in order to remove metal ions. In the case of manganese supplementation $MnCl_2 \cdot 4H_2O$ was added to 5×10^{-5} M.

Preparation of Cell Walls. The mycelium was collected by suction filtration, washed twice with cold tap water and once with distilled water, blotted between filter paper and weighed. An aliquot (5–10 g) was treated with 100 ml of 1% (w/v) dodecylsulfate at room temperature with continuous stirring for 4 h. The suspension was then frozen, thawed and homogenized by means of a Potter-Elvehjem homogenizer. After filtration, the procedure was repeated with the residue. The final debris was suspended in 25 ml of tap water, stirred for 10 min and filtered. This was repeated until the filtrate showed no

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absorption at 260 nm. The debris was then washed with absolute methanol and dried at room temperature. As specified under "Results", this material appeared to be free of cytoplasmic contaminants (ribose, certain amino acids, nucleic acids).

Fractionation of the Cell Walls. The cell wall powder was subjected to fractionation according to the scheme of Mahadevan and Tatum (1965). The first alkali soluble fraction (F 1) was precipitated by addition of two volumes of ethanol, the precipitate dialyzed against distilled water, collected by suction through a sinter funnel and dried in an exsiccator at room temperature. The resulting weight of the precipitate was taken for the amount of F 1. The following acid extraction yielded a soluble fraction, which was neutralized and analyzed (F 2). The second alkali soluble fraction was again precipitated, dialyzed and weighed as described above (F 3). The resulting residue was dialyzed against distilled water, filtered and dried at room temperature (F 4).

Hydrolysis of the Cell Wall. Hydrolysis of the cell walls or cell wall fractions was performed in sealed tubes under nitrogen by means of tenfold (v/w) 6 N HCl (for analysis of amino acids) or 4 N HCl (for analysis of sugars and aminosugars) at 100°C for 16 h. The hydrolysates were filtered and concentrated in vacuo at room temperature. The residue was taken up in 1 ml of distilled water and kept in ice until analysis.

Analytical Methods. Total carbohydrate was determined by the anthrone procedure of Loewus (1952) using a glucose standard. For qualitative sugar determination, thin layer plates of silica gel were irrigated with butanol – acetone – water (4:5:1, v/v) for 1 h, and after drying, with chloroform – acetic acid – water (10:7:1, v/v) in the same direction for 90 min (Zonneveld, 1971). Monosaccharides were detected by spraying with anisaldehyde-H_2SO_4 (Krebs et al., 1967). Glucose and galactose were assayed enzymatically with the appropriate sugar oxidases (Boehringer Mannheim, Germany). Mannose was determined as the difference between total carbohydrate and glucose plus galactose, since only these three monosaccharides were detected by thin layer chromatography. Total hexosamines were assayed by the Elson-Morgan procedure (Tracey, 1955). Individual hexosamines and amino acids were determined by means of an amino acid analyzer as reported previously (Kubicek et al., 1979) with the exception that the pH of the buffer for separation of neutral amino acids was 4.1 (instead of 4.25) to avoid overlapping of glucosamine and isoleucine. Protein in cell walls was extracted and determined as reported previously (Kubicek et al., 1979).

Results

Effect of Manganese Concentration

The effect on hyphal morphology and citric acid production of various concentrations of Mn^{2+} in the medium was examined. The addition of as little as 4×10^{-7} mol/l of manganese reduced the acid yield by 10% and produced the undesirable 'filamentous' type of hyphae (Table 1) together with the 'producing' type of hyphae. Concentrations of manganese of 10^{-6} M and higher produced only filamentous hyphae. Minimal acidogenesis was observed with 5×10^{-5} M manganese (16% of control).

Other strains *Aspergillus niger* (ATCC 11 414 or a low acidogenic wild strain from the institute collection) displayed the same response in morphology to various manganese concentrations, indicating that this behaviour was not a feature of high acid producing strains only.

Table 1. Effect of manganese[a] on citric acid production and hyphal morphology

Mn^{2+} added (M)	Citric acid after 150 h (M)	Morphology	
		filamentous	bulbous
0	0.286	–	+
1×10^{-8}	0.283	–	+
1×10^{-7}	0.269	–	+
4×10^{-7}	0.251	+	+
1×10^{-6}	0.133	+	–
1×10^{-5}	0.075	+	–
5×10^{-5}	0.044	+	–

[a] Added as $MnCl_2 \cdot 4H_2O$. Other experiments revealed that the effect was obtained regardless of the anion of the salt

The effect of manganese was not influenced by addition of zinc or iron at concentrations up to 10^{-4} M. High concentrations of iron (above 5×10^{-4} M) also produced filamentous growth in the absence of manganese. Copper, on the other hand, reduced the influence of manganese, when added in excess (10^{-4} – 10^{-3} M, depending on the concentration of manganese). This is of special interest since it is generally believed that copper acts primarily as an antagonist of iron (Schweiger, 1961).

These findings thus demonstrate that manganese is a responsible trace metal for the morphological pattern of *Aspergillus niger*. This coincides with the findings of Clark et al. (1966), who studied the influence of trace metal contaminants in citric acid fermentation of molasses with respect to morphology.

Morphological Development of Aspergillus niger

In the manganese sufficient (5×10^{-5} M) medium, the initial morphological feature of the outgrowth of the spore was germ tube formation, which occurred after 18 – 20 h. The germ tubes were thin, and had only very few branches. In contrast, manganese deficient development started with considerable spherical outgrowth of the spores prior to tube formation. The developing germ tubes were short and thick and produced squat bulbous cells (Fig. 1). Subsequently pellet formation occurred exhibiting the typical characteristics of citrate producing (manganese deficient) and poor citrate producing (manganese sufficient) morphological features, respectively (Fig. 2).

Cell Wall Isolation and Composition

Previous experiments had shown that there were no major differences in either cell wall composition or

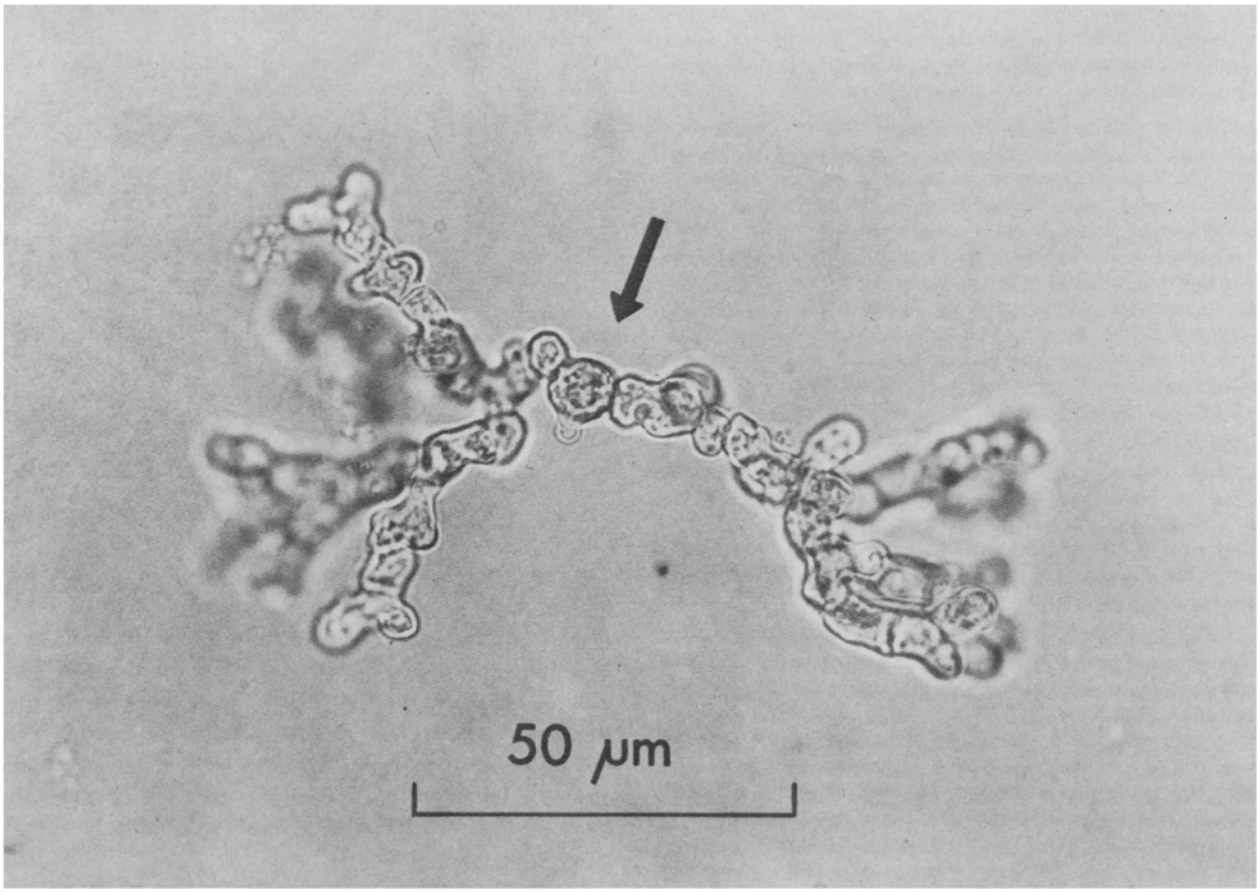

Fig. 1. Outgrowth from a spore of *Aspergillus niger* under conditions of manganese deficiency (30 h of incubation). Arrow indicates the swollen spore

morphology during growth with low or high Mn^{2+}. Studies on cell wall composition were therefore carried out with hyphae harvested at the end of the trophophase (55 h at high, 65 h at low Mn^{2+}). Cell walls, isolated from *Aspergillus niger* at different times during fermentation, accounted for the major portion of the fungal dry weight, namely 55 % at high and 61 % at low Mn^{2+}, respectively. The values were almost constant throughout the fermentation (± 4 %).

Initially some difficulties were encountered when hyphae from a medium high in manganese were used in the isolation of cell walls. This was due to considerable production of slime under these conditions. The slime bound tight to the hyphae, and could only be removed by treatment with boiling water. However, this treatment also extracted part of the α-glucan of the cell wall (Gold et al., 1973). Fortunately, the slime itself could be shown to contain very little glucose (less than 3 %), whereas the material from the cell wall, which could be solubilized with boiling water, consisted purely of glucose. Thus the glucose content of the extract was attributed to the cell wall.

Table 2. Composition of the complete wall of *Aspergillus niger* grown in the presence (Mn +) or absence (Mn −) of manganese ions

Constituents	Percent of cell wall dry weight	
	Mn +	Mn −
Neutral carbohydrates	80.75	64.95
Aminosugars	7.8	18.05
Proteins	2.9	2.4
Lipids	0.5	0.5
Recovery	92	86

Table 2 shows the chemical composition of the cell wall. Neutral carbohydrates accounted for the major portion. In agreement with previous reports on *Aspergillus niger* only glucose, galactose and mannose were detected as components of the wall. The absence of pentoses (especially ribose) indicates that the preparation was free of cytoplasmic contaminants. Lipid levels were very low in both cases, which is in contrast to

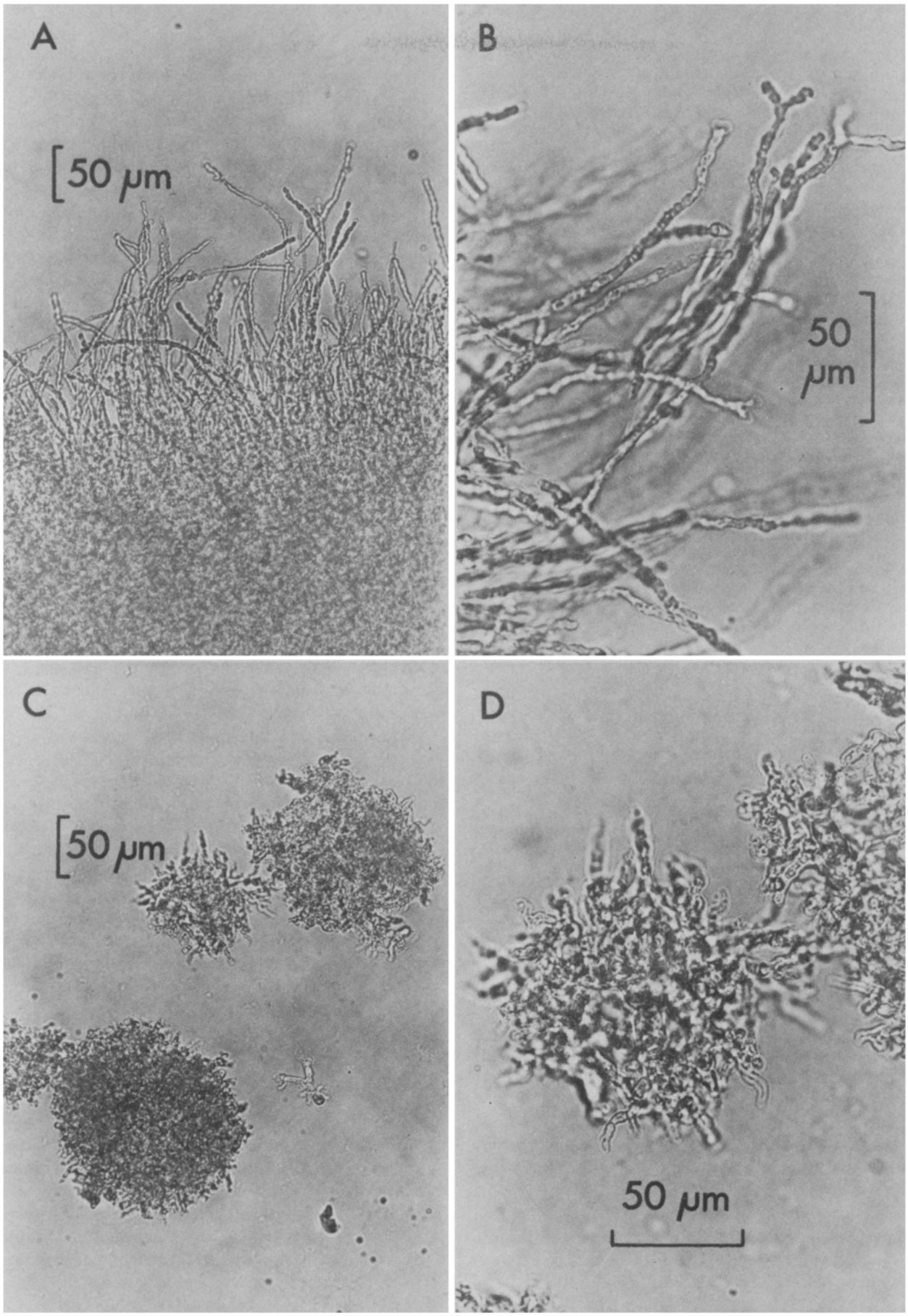

Fig. 2. Growth of *Aspergillus niger* in the presence (5×10^{-5} M) (**A** and **B**) and absence of manganese (**C** and **D**) at 50 h of incubation

Table 3. Distribution of monosaccharides, in % of total cell wall, in the fractions obtained by sequential extraction of hyphal walls from *Aspergillus niger* grown in the presence of 5×10^{-5} M Mn^{2+}. Fractions F 1 – F 4 are explained under "Materials and Methods", following the nomenclature of Mahadevan and Tatum (1965)

	F 1	F 2	F 3	F 4	Total
Glucose	34.2	17.3	18.0	–	69.5
Galactose	6.3	1.4	–	–	7.7
Mannose	3.1	0.5	–	–	3.6
Glucosamine	–	1.2	–	5.3	6.5
Galactosamine	1.3	–	–	–	1.3
Residue	5.1	4.6	0.3	1.5	11.4
Gravimetric analysis	50.0	25.0	18.2	6.8	100

Values for gravimetric analysis are means of ten separate experiments with a standard deviation of less than 12% of the given value. Monosaccharide and aminosugar values are means of three determinations carried out on separately hydrolyzed fractions. Residue means the difference between gravimetric and chemical analysis. Values for gravimetric analysis of F 2 are the differences between cell wall dry weight before and after acid extraction

Table 4. Distribution of monosaccharides, in % of total cell wall, in the fractions obtained by sequential extraction of hyphal walls from *Aspergillus niger* grown in the absence of manganese. Fractions F 1 – F 4 see under "Materials and Methods". See also footnote to Table 3

	F 1	F 2	F 3	F 4	Total
Glucose	26.3	28.5	7.0	–	61.8
Galactose	1.1	0.7	–	–	1.8
Mannose	0.7	0.7	–	–	1.4
Glucosamine	–	2.3	–	15.3	17.6
Galactosamine	0.5	–	–	–	0.5
Residue	4.4	7.8	–	4.7	16.9
Gravimetric analysis	33.0	40.0	7.0	20.0	100.0

some other reports on Aspergilli (Bull, 1970; Zonneveld, 1971). Glucosamine content was significantly increased under manganese deficient conditions. Minor levels of galactosamine were also found.

Composition of Cell Wall Fractions

To obtain further knowledge about the polymers of the cell walls, they were subjected to the fractionation scheme of Mahadevan and Tatum (1965).

The distribution of monosaccharides and aminosugars in hydrolysates of a series of extracts from manganese sufficient and deficient grown hyphal walls is shown in Table 3 and 4.

The first alkali-soluble fraction (F 1) contained glucose, galactose, mannose and galactosamine. This fraction accounted for 50 and 33% of the wall under manganese sufficient and deficient conditions, respectively. Manganese deficiency reduced the contents of all components listed above, but this reduction was mostly pronounced with galactose (6.3 to 1.1%, respectively). The nature of the galactose polymer is not known. Bardalaye and Nordin (1976) reported the presence of galactosaminogalactan in cell walls from *Aspergillus niger*. Using their method of purification, both galactosaminogalactan as well as galactoglucomannan could be isolated from the undialyzable ethanol precipitate of fraction 1, which together accounted for 83% of galactose and mannose in fraction 1.

The second, acid soluble fraction (F 2) contained glucose, galactose, mannose and glucosamine. This fraction accounted for a major part of the cell wall under manganese deficiency, mainly due to a significantly higher glucose content, as compared to the cell wall produced in the presence of manganese. No attempts were made to identify further the polymers in this fraction.

The second alkali soluble fraction (F 3) consisted purely of glucose. Treatment of the ethanol precipitated undialyzable material with a β-glucanase containing enzyme preparation from *Arthrobacter* sp. (Müllner and Röhr, 1974) liberated only glucose, whereas treatment with bacterial α-amylase had no effect. This is in accordance with the findings of Mahadevan and Tatum (1965) that F 3 specifically contains β-glucan. The amount of this fraction was significantly reduced under manganese deficiency.

The residue resulting from these treatments (F 4) yielded only glucosamine after hydrolysis, indicating the presence of chitin. A threefold higher level was found under manganese deficient conditions.

Amino acid composition of the two types of cell walls revealed no major differences – either qualitatively or quantitatively (Table 5). The absence of certain amino acids (esp. γ-aminobutyrate, ornithine, glutamine) which have been reported to accumulate in manganese deficient mycelia of *Aspergillus niger* (Kubicek et al., 1979), can be considered as a good criterium for the purity of the preparations and the absence of cytoplasmic components.

Effect of Copper

As reported above, addition of an excess of copper can induce the appearance of 'citric acid producing' morphology in the presence of manganese. Analysis of the chemical composition of cell walls, isolated from hyphae grown in the presence of 5×10^{-5} M Mn^{2+} and 10^{-4} M Cu^{2+} is given in Table 6. An excess of copper produces the same effect as manganese deficiency. Compared to manganese sufficient grown hyphae, elevated chitin, and reduced β-glucan as well as galac-

Table 5. Amino acid components of hyphal walls of *Aspergillus niger* grown in the presence (+) or absence (−) of 5×10^{-5} M manganese. Values are given as μmol/mg cell wall[a]

	(+)	(−)
Aspartate	0.032	0.019
Serine	0.025	0.024
Threonine	0.026	0.025
Glutamate	0.031	0.022
Proline	0.019	0.016
Glycine	0.027	0.026
Alanine	0.031	0.023
Valine	0.016	0.017
Methionine	0.002	0.0015
Isoleucine	0.015	0.012
Leucine	0.027	0.021
Phenylalanine	0.011	0.010
Tyrosine	0.012	0.012
Lysine	0.019	0.012
Arginine	0.023	0.018
Histidine	TR	TR
Total	0.316	0.2585

[a] TR only traces found

Table 6. Distribution of monosaccharides, in % of total cell wall, in the fractions obtained by sequential extraction of hyphal walls from *Aspergillus niger* grown in the presence of 5×10^{-5} M Mn^{2+} and 10^{-3} M Cu^{-2+}. Fractions F1–F4 seen under Materials and Methods. See also footnote to Table 3

	F1	F2	F3	F4	Total
Glucose	29.4	30.0	8.0	–	67.4
Galactose	1.4	1.1	–	–	2.5
Mannose	1.4	0.4	–	–	1.8
Glucosamine	–	3.1	–	14.2	17.3
Galactosamine	0.7	–	–	–	0.7
Residue	4.1	3.4	0.0	2.8	10.3
Gravimetric analysis	37.0	38.0	8.0	17.0	100.0

tose levels were found. No change in amino acid composition was observed.

Effect of Cycloheximide

Experiments showed that expression of filamentous morphology can be induced by the addition of manganese to a manganese deficient culture during trophophase (first 70 h). Simultaneous addition of cycloheximide and manganese, however, inhibits filamentous growth. When *Aspergillus niger* is grown in the presence of cycloheximide and manganese (10 mg/l and 5×10^{-5} M, respectively) biomass formation is severely inhibited (5% of control). The morphology, however, shows some striking parallelism to manganese deficient development (Fig. 3). Cell wall analysis of these hyphae

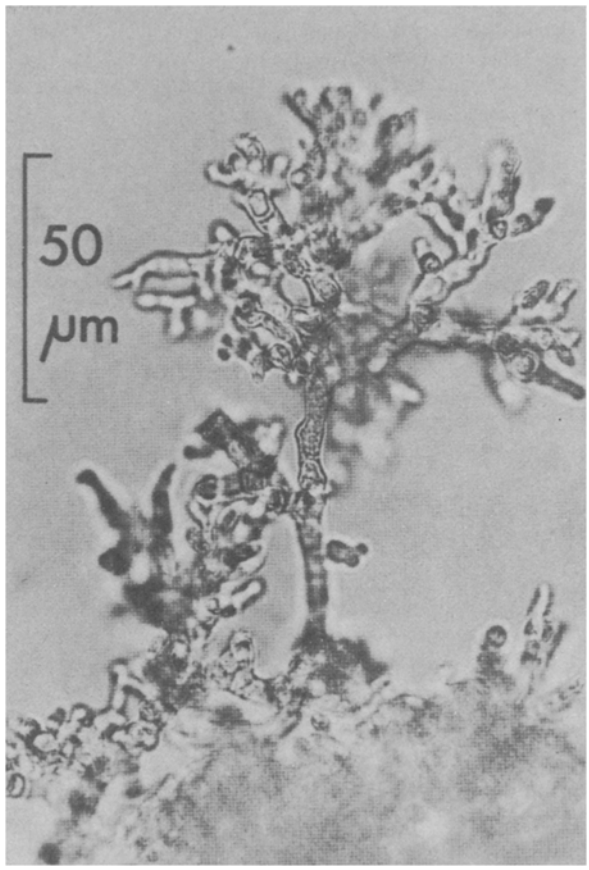

Fig. 3. Growth of *Aspergillus niger* in the presence of manganese (5×10^{-5} M) and cycloheximide (10 mg/l) (50 h of incubation)

also revealed elevated chitin and reduced β-glucan contents. Fraction 1 and 2, however, rather resembled the composition of manganese sufficient hyphae. Galactose levels were not influenced as severely as during manganese deficiency (Table 7).

Discussion

The present work demonstrates that manganese is an essential trace metal for filamentous growth of *Aspergillus niger*. In the absence of manganese (i.e. estimated levels lower than 10^{-8} M) abnormal development occurs, which has some parallelism to giant cell germination at elevated temperatures (Anderson and Smith, 1972) and the so called 'yeast-like' growth of *Aspergillus parasiticus* (Detroy and Ciegler, 1971). This type of growth produces high amounts of citric acid in *Aspergillus niger*, whereas filamentous growth is accompanied by lower acid yields.

Manganese sufficient and deficient grown hyphae differ considerably with regards to cell wall composition. Under manganese deficiency elevated chitin

Table 7. Distribution of monosaccharides, in % of total cell wall, in the fractions obtained by sequential extraction of hyphal walls from *Aspergillus niger* grown in the presence of 5×10^{-5} M Mn^{2+} and 10 mg/l cycloheximide. Fractions F 1 – F 4 see under "Materials and Methods". See also fomtnote to Table 3

	F 1	F 2	F 3	F 4	Total
Glucose	31.6	19.4	5.3	–	56.3
Galactose	4.7	0.8	–	–	5.5
Mannose	2.2	0.8	–	–	3.0
Glucosamine	–	2.1	–	18.7	20.8
Galactosamine	0.7	–	–	–	0.7
Residue	–	8.1	–	5.6	13.7
Gravimetric analysis	39.2	31.2	5.3	24.3	100.0

and reduced β-glucan contents are observable. Also, a reduction in alkali soluble galactose polymers can be detected. However, the role of the latter compounds in cell wall morphology is unclear. Addition of cycloheximide to manganese sufficient growth medium results in a very similar type of growth as manganese deficiency. Analysis of cell wall composition of these hyphae reveals elevated chitin and reduced β-glucan contents, but the influence on galactose levels is not so pronounced. The conclusion is thus drawn that the relative proportion of chitin to β-glucan may be responsible for the hyphal shape in *Aspergillus niger*, and that a disturbance in protein metabolism brings about an alteration in this ratio. It should be mentioned that cycloheximide-induced increased branching of hyphae in *Aspergillus nidulans* (Sternlicht et al., 1973), yeast-like cell formation in *Paracoccidioides brasiliensis* (Kanetsuna et al., 1969) as well as giant cell formation in *Penicillium citrinum* (Katoh et al., 1978) could be attributed to increased chitin and reduced β-glucan levels.

The fact that inhibition of protein synthesis brings about changes in cell wall composition is well known for several fungi (Farkas, 1979). However, the mode of influence is still unclear. It has been suggested that inhibition of glycoprotein turnover may lead to a loss of hyphal polarity and thus increased branching and chitin synthesis. On the other hand, it has been observed (Kubicek et al., 1979; Habison et al., 1979) that manganese deficiency results in elevated cellular concentrations of fructose-6-phosphate and glutamine, the initial metabolites for chitin synthesis. As it is known that the respective enzyme from fungi (fructose-6-phosphate glutamine-aminotransferase) has rather low affinities for its substrates ($K_{m\,F6P}$ 1–2 mM; K_{mGln} 0.7 mM; Gooday, 1978), elevated precursor concentrations may lead to increased metabolic flux in the direction of chitin synthesis as has been suggested by Chattaway et al. (1973) for *Candida albicans*.

Reduced β-glucan was mostly explained in terms of high turnover rates of the appropriate synthases. This may also be applicable in the present case. The experiments with cycloheximide make it less likely that a manganese-dependent β-glucan synthase may be involved. Although the presence of a Mn^{2+} requiring β-glucan-synthase has been reported in *Saccharomyces cerevisiae* (Balint et al., 1976), this could not be confirmed with other fungi (Wang and Bartnicki-Garcia, 1966; San Blas, 1979).

However, there may be other cases where manganese could act as a cofactor. Mannosyltransferase from *Mucor rouxii*, for example, is manganese dependent (Ruiz-Herrera and Gutierrez, 1978). In the present case, galactose levels from fraction 1 were specifically dependent on the presence of manganese, but minor changes were observed during cycloheximide treatment. It may be assumed that one or more of the steps leading to galactose-polymers in *Aspergillus niger* requires manganese.

The data provide evidence for the linkage between *Aspergillus niger* morphology and cell wall composition. It further demonstrates that an impairment of protein or RNA-turnover due to manganese deficiency, which is considered to have a regulatory function in citric acid accumulation (Kubicek et al., 1979; Habison et al., 1979; Kubicek and Röhr, 1977), might also be the key factor in the expression of the 'citric acid producing' type of morphology.

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