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 Mn²⁺ (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 \(\beta\)-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 \(\beta\)-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 (Detry 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 \(\alpha\)-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 MnCl₂ · 4H₂O was added to \(5 \times 10^{-5}\) 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...
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 (F1) 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 F1. The following acid extraction yielded a soluble fraction, which was neutralized and analyzed (F2). The second alkali soluble fraction was again precipitated, dialyzed and weighed as described above (F3). The resulting residue was dialyzed against distilled water, filtered and dried at room temperature (F4).

Hydrolysis of the Cell Walls. 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 - acetic acid - 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 50 min (Zonneveld, 1971). Monosaccharides were detected by spraying with anisaldehyde - H₂SO₄ (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 Mn²⁺ in the medium was examined. The addition of as little as 4 x 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 x 10⁻⁶ M manganese (16% of control).

Other strains Aspergillus niger (ATCC 11414 or a low acidogenic wild strain from the institute collection) displayed the same response in morphology to various manganese concentrations, indicating that this be-

<table>
<thead>
<tr>
<th>Mn²⁺ added (M)</th>
<th>Citric acid after 150 h (M)</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.286</td>
<td>-</td>
</tr>
<tr>
<td>1 x 10⁻⁸</td>
<td>0.283</td>
<td>-</td>
</tr>
<tr>
<td>1 x 10⁻⁷</td>
<td>0.269</td>
<td>-</td>
</tr>
<tr>
<td>4 x 10⁻⁷</td>
<td>0.251</td>
<td>+</td>
</tr>
<tr>
<td>1 x 10⁻⁶</td>
<td>0.133</td>
<td>+</td>
</tr>
<tr>
<td>1 x 10⁻⁵</td>
<td>0.075</td>
<td>+</td>
</tr>
<tr>
<td>5 x 10⁻⁵</td>
<td>0.044</td>
<td>-</td>
</tr>
</tbody>
</table>

* Added as MnCl₂ - 4H₂O. Other experiments revealed that the effect was obtained regardless of the anion of the salt.

Morphological Development of Aspergillus niger

In the manganese sufficient (5 x 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