Factors Influencing the Production of Cellulase by
Aspergillus fumigatus (Fresenius)

By J. C. STEWART* AND JOCELYN B. PARRY
Department of Biological Sciences, Coventry (Lanchester) Polytechnic, Priory Street,
Coventry CV1 5FB, U.K.

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During growth in liquid culture containing a single cellulosic or non-cellulosic carbon source, a newly isolated strain of Aspergillus fumigatus released cellulases into the medium; the amounts produced depended on the nitrogen source, the type and concentration of the carbon source, pH and temperature. Extracellular cellulolytic activity was still increasing after incubation for 60 d with 1% (w/v) CF11 cellulose, (NH₄)₂SO₄ as nitrogen source and a starting pH of 7. The activities of the new isolate were compared with those of A. fumigatus IMI 143864 and Trichoderma reesei QM6a (ATCC 13631) and it was shown to be a good producer of β-glucosidase.

INTRODUCTION

Cellulose is abundant in municipal, agricultural and forestry wastes. Given an economical process it could be converted into cellobiose or glucose which could form utilisable substrates for the production of single cell protein or other fermentation products. Enzymic hydrolysis is one way of achieving this conversion.

Important amongst cellulolytic micro-organisms are the filamentous fungi, some of which are thermophilic whilst others are thermotolerant (Cooney & Emerson, 1964). Such organisms have high metabolic rates and digest cellulosic wastes quite rapidly. Composts are good sources of these organisms (Chang, 1967; Fergus, 1964; Kane & Mullins, 1973) because of the high temperatures reached during the microbial degradation of organic matter. Basu (1948) investigated the ability of a strain of Aspergillus fumigatus to break down jute cellulose and Reese & Levinson (1952) compared the cellulolytic ability of A. fumigatus with that of many other organisms. Chang (1967) isolated A. fumigatus from wheat straw compost and showed that it could digest filter paper and hemicellulose.

In the present study we have isolated a strain of A. fumigatus from garden compost, tested its ability to grow and produce cellulase on various carbohydrates including soluble and insoluble celluloses, and examined the effects of nitrogen source, pH and temperature. We have also compared the enzyme activities, growth and cellulose decomposition of this organism with those of A. fumigatus IMI 143864 and Trichoderma reesei QM6a (ATCC 13631).

METHODS

Organisms. The strain of Aspergillus fumigatus Fresenius was isolated from garden compost by enrichment culture in a mineral salts/cellulose medium followed by repeated subculture on to mineral salts/cellulose agar plates. It was identified at the Commonwealth Mycological Institute, Kew, U.K., and designated IMI 246651. Aspergillus fumigatus IMI 143864 and Trichoderma reesei QM6a (ATCC 13631), used in comparative studies, were obtained from the appropriate culture collections.
Media. Stock cultures were maintained on malt extract agar (MEA; Difco) or potato dextrose agar (PDA; Difco) slants in 25 ml screw-cap bottles at 37 °C. The enrichment culturing was in modified Czapek-Dox medium in which the sucrose was replaced by Whatman CF 11 cellulose at a concentration of 1% (w/v). The medium was modified during the course of the study and had the following final composition (per litre): (NH₄)₂SO₄, 2.5 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.5 g; FeSO₄·7H₂O, 5 mg; MnSO₄·H₂O, 1.5 mg; ZnSO₄·7H₂O, 1.4 mg; CoCl₂·6H₂O, 2 mg; adjusted to pH 7. For making plates, 2% (w/v) agar was added. In some experiments, alternative nitrogen sources (NaNO₃, NH₄NO₃, urea, peptone or biuret) were used, at concentrations calculated to supply equivalent amounts of nitrogen; in others, the pH was controlled by preparing the medium (without KH₂PO₄) in 0.1 M-potassium phosphate buffer of the required pH. Incubations were carried out in a rotary shaker (120 rev. min⁻¹) in 250 ml Erlenmeyer flasks containing 100 ml medium sterilized by autoclaving at 121 °C for 15 min.

Trichoderma reesei QM6a was grown on the medium of Mandels & Reese (1956).

Inocula. Slants showing heavy spore production after incubation for 2–3 d on PDA in 25 ml screw-cap bottles were used as sources of inocula. To each slant 10 ml sterile water containing 0.1% (v/v) Tween 80 and 8–10 undrilled glass beads (BDH) were added, and the spores were dislodged by gentle shaking. The resulting suspensions were added to the flasks to give 10⁷ to 10⁸ viable spores per 100 ml medium. Viable spore counts were made by plating 0.1 ml of suitably diluted spore suspensions on to MEA plates containing 0.08% (w/v) deoxycholate (Sigma) and incubating at 37 °C for 2 d.

Preparation of culture filtrates. Medium was separated from unused cellulose and mycelium by filtering through sintered glass filters.

Growth studies. Flask contents were filtered through sintered glass filters (porosity 1) and washed. Dry weight was determined after heating at 80 °C overnight. Protein content of the dried flask contents was determined by heating 100 mg samples with 100 ml 0.1% (w/v) sodium dodecyl sulphate (SDS) at 100 °C for 3 min; after centrifuging, protein was determined by the Lowry method.

Enzyme assays. Exoglucanase (EC 3.2.1.91) activity was measured either with filter paper by the method of Mandels & Weber (1969) or with cotton dewaxed by the method of Halliwell (1957). For the latter assay, the reaction mixture consisted of 1 ml culture filtrate and 20 mg dewaxed Texas cotton in 1 ml 0.1 M-acetate buffer pH 5 containing 0.02% (w/v) NaN₃; after incubation at 37 °C for 18 h, the soluble sugar produced was determined by the phenol/H₂SO₄ method of Dubois et al. (1956). Endoglucanase (EC 3.2.1.4) activity was measured by the method of Matsumoto (1974) using carboxymethylcellulose (CMC). β-Glucosidase (EC 3.2.1.21) activity was measured by the method of Okada et al. (1967) using p-nitrophenyl-β-D-glucopyranoside.

One unit of enzyme (EU) is defined as the amount catalysing the production of 1 μmol glucose h⁻¹. In all cases, enzyme activity is expressed as EU (ml culture filtrate)⁻¹.

Chemicals. Carboxymethylcellulose (CM-cellulose, sodium salt, medium viscosity), and p-nitrophenyl-β-D-glucopyranoside were from Sigma. CC4I microgranular cellulose, CF11 long-fibred cellulose and CF11 medium-fibred cellulose were all from Whatman. Avicel PH101 was from Honeywill & Stein Ltd, Wallington, U.K. The cotton floc was provided by The Rowett Research Institute, Aberdeen.

RESULTS AND DISCUSSION

Effect of culture conditions on growth and cellulase production by A. fumigatus IMI 246651

When ammonium salts were used as nitrogen source the pH value of the medium always decreased during growth (Table 1); this was most pronounced with ammonium sulphate.

Table 1. Effect of nitrogen source on the production of cellulases by A. fumigatus IMI 246651

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Endoglucanase (EU ml⁻¹)</th>
<th>Exoglucanase (EU ml⁻¹)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>65</td>
<td>4.7</td>
<td>4.0</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>16</td>
<td>1.5</td>
<td>7.6</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>96</td>
<td>4.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Biuret</td>
<td>17</td>
<td>1.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Urea</td>
<td>6</td>
<td>0.9</td>
<td>8.7</td>
</tr>
<tr>
<td>Peptone</td>
<td>8</td>
<td>2.0</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Flasks containing 100 ml medium with 1% CF11 cellulose and different nitrogen sources were inoculated with 7·0 × 10⁷ spores and incubated at 37 °C for 16 d. Exoglucanase was measured by the filter paper method. Each result represents the mean of determinations on duplicate flasks.
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Cellulase production, however, was always higher when ammonium salts were used. The effect of pH on cellulase production, growth and cellulose breakdown was investigated further by growing the organism on a medium with sufficient buffering capacity to resist pH changes in the short term. The results are shown in Fig. 1(a, b). It is always difficult to measure the
growth of fungi on insoluble substrates as the separation of mycelium and substrate remaining is a problem. Fungal growth, measured as mycelial protein, was greatest at pH 6, but weight loss of the dry insoluble matter remaining after incubation (which indicates approximately the cellulose converted to soluble sugars and CO₂) was almost constant in the range pH 3 to 6 and only decreased above pH 6 (Fig. 1b). The maximum production of exo- and endoglucanases corresponded to the optimum growth at pH 6 (Fig. 1a), though the extracellular enzymes were most efficient in the range pH 4.5 to 5.5 (Fig. 1c). Similarly, although maximum enzyme production, growth and weight loss occurred in the range 35 to 45 °C (Fig. 2a, b), the extracellular enzymes had temperature optima of 60 °C under the assay conditions, though this may be different with the longer incubation times used for growing the cultures. Trivedi & Rao (1979) cultured their strain of A. fumigatus at 28 °C but the present results indicate that our isolate of A. fumigatus is thermotolerant and produces more enzyme at higher temperatures.

The effect of different carbon sources is shown in Table 2. In general, more cellulase activity was produced on insoluble cellulose sources, the more amorphous celluloses giving higher yields of extracellular enzyme. Simple soluble sugars did not fully suppress the production of cellulases in this strain, unlike the strain used by Trivedi & Rao (1979), but lactose did not act as an inducer as it does in T. reesei (Mandels & Reese, 1956). Variation in the cellulose concentration indicated that apparently higher yields of cellulase were obtained at lower concentrations of the substrate (Fig. 3), possibly due to derepression of enzyme synthesis (Clarke & Lilly, 1969); lower yields at higher substrate concentrations could be due to absorption of the enzymes on to cellulose.

**Comparison with other organisms**

The new isolate of A. fumigatus (IMI 246651) was compared with A. fumigatus IMI 143864 and T. reesei QM6a to assess its effectiveness in producing enzymes and breaking down cellulose. The cultures were incubated for 60 d to minimize the effect of different inoculum sizes and to determine any long-term cultural effects.

The new isolate of A. fumigatus grew at a similar rate to T. reesei over the 60 d incubation period (Fig. 4b). The growth of A. fumigatus IMI 143864 was always much slower, with little

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**Table 2. Effect of carbon source on the production of cellulases by A. fumigatus IMI 246651**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Weight loss (%)</th>
<th>Endoglucanase (EU ml⁻¹)</th>
<th>Exoglucanase (EU ml⁻¹)</th>
<th>β-Glucosidase (EU ml⁻¹)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>-</td>
<td>9</td>
<td>1.73</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Cellobiose</td>
<td>-</td>
<td>15</td>
<td>1.51</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>12</td>
<td>0.33</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>8</td>
<td>1.08</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>-</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF1</td>
<td>51</td>
<td>40</td>
<td>0.50</td>
<td>1.73</td>
<td></td>
</tr>
<tr>
<td>CFll</td>
<td>50</td>
<td>42</td>
<td>0.53</td>
<td>1.51</td>
<td></td>
</tr>
<tr>
<td>CC41</td>
<td>51</td>
<td>34</td>
<td>0.40</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>Avicel</td>
<td>68</td>
<td>35</td>
<td>0.45</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>Cotton</td>
<td>11</td>
<td>37</td>
<td>0.25</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>Cotton wool</td>
<td>5</td>
<td>13</td>
<td>0.13</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Sawdust</td>
<td>3</td>
<td>23</td>
<td>0.14</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Cardboard</td>
<td>67</td>
<td>36</td>
<td>0.18</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Newspaper</td>
<td>40</td>
<td>35</td>
<td>0.18</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

* Not determined due to high concentrations of reducing sugar in the culture filtrate.
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Fig. 3. Effect of concentration of CF11 cellulose (○) and Avicel (●) on cellulase production in cultures of *A. fumigatus* IMI 246651. Flasks containing 100 ml medium were inoculated with $5.9 \times 10^7$ spores and incubated at 37°C for 30 d. Exoglucanase was determined by the cotton method. Each point represents the mean of determinations on duplicate flasks.

Fig. 4. Variation with incubation time of weight loss and total mycelial protein in cultures of *T. reesei* QM6a (○), *A. fumigatus* IMI 246651 (●) and *A. fumigatus* IMI 143864 (□). Flasks containing 100 ml medium with 1% (w/v) CF11 cellulose were inoculated with $5.4 \times 10^8$, $2.9 \times 10^{10}$ and $3.2 \times 10^7$ spores, respectively, and incubated at 28°C (*T. reesei*) or 37°C (*A. fumigatus*). Each point represents the mean of determinations on duplicate flasks.

increase in mycelial protein after 20 d; in this respect, the growth pattern was similar to that of a strain of *A. fumigatus* observed by Trivedi & Rao (1979) in which growth at 28°C on $(NH_4)_2SO_4$ and 0.5% Sigma cellulose was complete after 12 d.

Much more endoglucanase was produced by *T. reesei* than by either of the *A. fumigatus* strains, the activity of *T. reesei* being eight times higher than that of the new *A. fumigatus*.
Fig. 5. Variation with incubation time of cellulase production in cultures of *T. reesei* QM6a (○), *A. fumigatus* IMI 246651 (●) and *A. fumigatus* IMI 143864 (□). Flasks containing 100 ml medium with 1% (w/v) CF11 cellulose were inoculated with $5.4 \times 10^8$, $2.9 \times 10^9$ and $3.2 \times 10^8$ spores, respectively, and incubated at 28°C (*T. reesei*) or 37°C (*A. fumigatus*). Exoglucanase was determined by the cotton method. Each point represents the mean of determinations on duplicate flasks.

isolate after 60 d incubation (Fig. 5a). With *T. reesei*, endoglucanase production was almost linear up to 35 d after a short initial lag period. With the two *A. fumigatus* strains, production was almost linear over the whole course of incubation but the rate of increase was much lower than with *T. reesei*. The exoglucanase activity of *T. reesei* was always greater than that of the *A. fumigatus* strains, though the new isolate (IMI 246651) produced greater activities than *A. fumigatus* IMI 143864, particularly in the long term (Fig. 5b). Previously, *T. reesei* was found to be a poor producer of β-glucosidase (Sternberg, 1976), and compared with the two *A. fumigatus* strains this was confirmed (Fig. 5c). The new isolate produced 14 times more β-glucosidase activity, at its maximum level, than *T. reesei*, although there was a lag period of 20 d before extracellular β-glucosidase could be demonstrated. Trivedi & Rao (1979) found with their strain of *A. fumigatus* that maximum enzyme synthesis was attained after 12 d, with no increase between 12 and 20 d. This timing is similar to that found with *A. fumigatus* IMI 143864 but quite different from that for the new isolate of *A. fumigatus* (IMI 246651) and *T. reesei*. The enzyme activities achieved in 12 d by the strain of Trivedi & Rao (1979) were higher for β-glucosidase and exoglucanase, but lower for endoglucanase, than in *T. reesei* or the new isolate of *A. fumigatus*, the endoglucanase activities being similar to those of *A. fumigatus* IMI 143864.

The dry weight losses with *T. reesei* and the new isolate of *A. fumigatus* were similar over 60 d incubation, both organisms giving an 80–90% loss in weight; the weight loss with *A. fumigatus* IMI 143864 was always lower (Fig. 4a). Approximately 30% of the cellulose was
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consumed in 12 d by the *A. fumigatus* strain of Trivedi & Rao (1979), but no further breakdown was found over the observed period of 20 d. Longer term growth studies are required to see if this strain is capable of more extensive cellulolysis. The similarity in the extent of cellulose breakdown by *T. reesei* and the new isolate of *A. fumigatus* may be a consequence of the production of roughly equivalent amounts of exoglucanase activity. Alternatively, the breakdown might be rate-limited by one of the other activities, i.e. endoglucanase activity in the case of the new isolate of *A. fumigatus*, or β-glucosidase in the case of *T. reesei*. Since each culture filtrate contains an excess of one of these two activities, and since cellulases are known to act synergistically in admixture (Wood, 1975), it should be possible to distinguish between these two possibilities by examining the effect of mixed culture filtrates of *T. reesei* and *A. fumigatus* on the breakdown of cellulose.

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REFERENCES


