Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicylic acid reagent method

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Cellulolytic fungi can easily be screened within 2 d for the production of cellulolytic enzymes by staining with Congo red, or by measuring the amount of reducing sugar (glucose) produced with the dinitrosalicylic acid reagent method. Endoglucanase activity is visible in carboxymethyl cellulose agar plates after staining with Congo red, and fixing with HCl or NaOH. This method is essentially based on the interaction of Congo red with intact β-(1-4)-β-glucans in carboxymethyl cellulose. Endoglucanase and exoglucanase activities of cellulases are quantitatively measured with the dinitrosalicylic acid reagent method. The enzymatic activity detected with Congo red is compared with that obtained by the dinitrosalicylic acid reagent method.

Scientific investigations on cellulases have intensified over the last few years, mainly because of the world-wide interest in exploiting renewable resources of biomass as a source of chemicals and liquid fuels (Mandels 1982). It has become important therefore to determine cellulolytic micro-organisms. It has been clearly shown thus far that the three enzymes involved in the degradation of cellulose, are β-glucosidase (EC.3.2.1.21), endo-β-(1-4)-d-glucanase (EC.3.2.1.4), and exo-β-(1-4)-d-glucanase (EC.3.2.1.91) (Mandels 1982). Several methods have been suggested for detecting cellulolytic activity in different micro-organisms (Hankin & Anagnostakis 1977; Smith 1977; Mahasneh & Stewart 1980). These methods are both tedious and insensitive.

The recent finding that Congo red shows a strong interaction with polysaccharides with contiguous β-(1-4)-bound-D-glucopyranosyl units provides the basis for a sensitive assay for detecting colonies of cellulase-producing bacteria (Teather & Wood 1982). An earlier method is used to determine reducing sugar produced enzymatically during the hydrolysis of carboxymethyl cellulose (Wood 1980).

This paper examines the use of Congo red to detect cellulase-producing fungi.

Materials and Methods

Chemicals

The granulated agar came from BBL Ltd, carboxymethyl cellulose (CMC, sodium salt, medium viscosity) from Sigma, and the Congo red from Merck. All other chemicals were of reagent grade and were obtained from Merck.

Organisms

Trichoderma reesei QM9414 (a high cellulase producer) was obtained from ATCC Ltd. Trichoderma harzianum, Trichotheceum roseum, Aspergillus ochraceus and Penicillium italicum
were from the fungal collection centre of the Marmara Research Institute. *Neurospora crassa* and *A. niger* were isolated locally.

**CULTURE MEDIA**

All fungi were initially grown on PDA plates. The medium contained (g/l): potatoes, 300; glucose, 20; agar, 15. The modified salt solution, originally defined by Mandels (1975) contained (g/l in distilled water): (NH$_4$)$_2$SO$_4$, 1.4; urea, 0.3; KH$_2$PO$_4$, 2; CaCl$_2$, 0.3; MgCl$_2$, H$_2$O, 0.3; FeSO$_4$.7H$_2$O, 0.005; MnSO$_4$.H$_2$O, 0.016; ZnCl$_2$.H$_2$O, 0.014; CoCl$_2$.6H$_2$O, 0.002; the pH value of the solution was 5. For the cellulolytic fungi test, 1% CMC, 1.5% agar and 1 ml of Triton-X-100 were also added to the salt solution (1 l).

**INOCULATION MEDIA**

Conidia, 10$^7$/ml, were plated in the centre of Petri dishes containing 1% CMC agar medium, and incubated at 26°C.

**VISUALIZATION OF ZONES OF CMC HYDROLYSIS**

After incubation at 26°C for 2 or 5 d, Petri dishes containing 1% CMC agar media were flooded with an aqueous solution of Congo red (1% Congo red in distilled water) and shaken at 50 rev/min for 15 min on a Nuve SL 350 shaker. The Congo red solution was then poured off, plates were further flooded with N NaCl and shaken again at 50 rev/min for 15 min. The fungal growth was stopped by flooding the

**Table 1. Characteristics of cellulolytic fungi**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Zone diameter (cm) of hydrolysis and days required to degrade CMC</th>
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</thead>
<tbody>
<tr>
<td><strong>Trichothecium roseum</strong></td>
<td>1.0 3.7</td>
</tr>
<tr>
<td><strong>Trichoderma harzianum</strong></td>
<td>2.1 4.3</td>
</tr>
<tr>
<td><strong>Neurospora crassa</strong></td>
<td>1.2 3.6</td>
</tr>
<tr>
<td><strong>Aspergillus niger</strong></td>
<td>2.5 4.2</td>
</tr>
<tr>
<td><strong>Aspergillus ochraceus</strong></td>
<td>1.7 4.0</td>
</tr>
<tr>
<td><strong>Penicillium italicum</strong></td>
<td>1.3 3.8</td>
</tr>
</tbody>
</table>

Zone diameters are averages of seven replicates, 10$^7$ conidia/ml plated.

CMC agar plates with N HCl which changed the dye colour to blue-violet (pH 0.1) and further inhibited enzymatic activity, or with N NaOH (pH 13.3), which slightly changed the dye colour to brownish-red and also inhibited enzymatic activity.

**ENZYME ASSAYS**

Cellulase activity was measured colorimetrically (Miller 1959) using either carboxymethyl cellulose (1% CMC in a 50 mmol/l Na-citrate buffer at pH 4.5) or Whatman No. 1 filter paper (1 x 6 cm strips (50 mg)) as substrate (Mandels et al. 1976).

**Results and Discussion**

All fungi examined produced zones of hydrolysis in CMC agar plates within 2 d (Table 1). Particularly, *Trichoderma harzianum* and *N. crassa* showed high activity against CMC

**Table 2. Hydrolysis of CMC and filter paper by a complete cellulase from the following fungi**

<table>
<thead>
<tr>
<th>Degradation of CMC (mg glucose liberated)</th>
<th>Degradation of filter paper (mg glucose liberated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organism</strong></td>
<td><strong>Days</strong></td>
</tr>
<tr>
<td><strong>Days</strong></td>
<td><strong>Days</strong></td>
</tr>
<tr>
<td><strong>Trichothecium roseum</strong></td>
<td>1 0.010 0.046 0.225 0.510 0.823 0.063 0.199 0.276 0.560 0.868</td>
</tr>
<tr>
<td><strong>Trichoderma harzianum</strong></td>
<td>1 0.120 0.427 0.510 0.627 0.877 0.147 0.550 0.661 0.727 0.885</td>
</tr>
<tr>
<td><strong>Neurospora crassa</strong></td>
<td>1 0.425 0.503 0.610 0.794 0.968 0.487 0.620 0.762 0.870 0.974</td>
</tr>
<tr>
<td><strong>Aspergillus niger</strong></td>
<td>1 0.180 0.307 0.536 0.812 0.937 0.200 0.338 0.739 0.842 0.950</td>
</tr>
<tr>
<td><strong>Penicillium italicum</strong></td>
<td>1 0.023 0.068 0.338 0.610 0.825 0.095 0.203 0.616 0.817 0.866</td>
</tr>
<tr>
<td><strong>Aspergillus ochraceus</strong></td>
<td>1 0.021 0.062 0.110 0.280 0.843 0.043 0.137 0.346 0.611 0.878</td>
</tr>
<tr>
<td><strong>Penicillium italicum</strong></td>
<td>1 0.052 0.120 0.280 0.398 0.714 0.078 0.137 0.300 0.450 0.780</td>
</tr>
</tbody>
</table>

Figures are averages of seven replicates, 10$^7$ conidia/ml plated.
Screening for cellulolytic fungi

Fig. 1. Hydrolysis of CMC and filter paper by cellulase preparation from *Trichoderma reesei*. Whatman no. 1 filter paper was cut into $1 \times 6$ cm strips (50 mg). A 50 mmol/l Na-citrate buffer at pH 4.5 was used and incubated at 50°C for 1 h. Saccharification = reducing sugar (g) $\times 0.9$/substrate (g) $\times 100$ (Chahal 1985). +, FP, endoglucanase, exoglucanase; O, CMC, endoglucanase; □, exoglucanase.

(diameter of zone of clearing $>2$ cm). *Trichothecium roseum*, *Trichoderma reesei*, *A. ochraceus* and *P. italicum* exhibited lower activity against CMC (diameter of zone of clearing 1–2 cm). Zones of all strains except *P. italicum* reached $4 \pm 0.4$ cm in 5 d. When the cellulase activity of fungi was tested quantitatively with the dinitrosalicyclic acid reagent method, distinct variations were obtained in the conversion of CMC and filter paper to glucose by different fungi (Table 2). In this experiment, the highest activity against CMC was exhibited by *Trichoderma reesei*, *N. crassa* and *Trichoderma harzianum* (Table 2), whereas *Trichothec. roseum*, *A. niger*, *A. ochraceus* and *P. italicum* showed relatively low activity (Table 2).

The results obtained with Congo red and with the dinitrosalicyclic acid reagent method were very much the same, with one exception. Although *Trichoderma reesei* produced only a 1–2 cm zone diameter of CMC hydrolysis, it gave very high glucose production in 2 d (0.503 mg/ml). This was probably because the vegetative growth rate of *Trichoderma reesei*, even on PDA medium, is relatively slow (Table 1), but the density of endoglucanase is probably high (Fig. 1).

Figure 2 shows CMC agar plated with *Trichoderma harzianum*, stained with Congo red and fixed with either $\equiv$ HCl or $\equiv$ NaOH. Both chemicals stop fungal growth and enhance the contrast between the halo and the background. Halo formation on CMC agar plates results from cleavage of CMC into fragments smaller than cellohexaose to which Congo red does not bind. Moreover, halos could result from cleavage of CMC into fragments small enough to be washed out of the plates during the staining procedure. In either case, only endoglucanase.

![Fig. 2. CMC agar plates stained with 1% Congo red. *Trichoderma harzianum* was allowed to grow for 2 d on CMC agar plates. Zones of CMC hydrolysis are clearly visible. a, A CMC plate only washed with $1 \equiv$ NaCl; b, after washing with $1 \equiv$ NaCl, it was fixed with $1 \equiv$ NaOH; c, after washing with $1 \equiv$ NaCl, it was fixed with $1 \equiv$ HCl.](image-url)
activity would be expected to produce a zone of hydrolysis (Wood 1980). All fungi used in this work effectively produce true cellulolytic activity (Table 2). Among the fungi studied here, *Trichoderma reesei*, *N. crassa* and *Trichoderma harzianum* produce high yields of cellulases, and hence convert more cellulose (filter paper) to glucose. These three organisms start from the first day of incubation to produce high yields of cellulases that continuously increase. *Aspergillus niger*, *A. ochraceus*, *P. italicum* and *Trichothec. roseum*, however, produce low levels of enzymes at first and these gradually increase. On the fifth day, in terms of glucose production, the difference between all fungi studied here became marginal.

In conclusion, the Congo red method coupled with the dinitrosalicylic acid reagent method can clearly be used for screening cellulase-producing fungal strains. Although the Congo red procedure looks qualitative it gives a zone diameter of hydrolysis of CMC that truly corresponds to the results obtained with the dinitrosalicylic acid reagent method. Therefore, the Congo red procedure can be used in a rapid, sensitive and reproducible way for screening cellulase-producing fungi.

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References


