Extracellular Cellulase Production by *Sporocytophaga myxococcoides* NCIB 8639

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*Sporocytophaga myxococcoides* NCIB 8639 utilized a number of cellulosic substrates and produced extracellular carboxymethylcellulase and activity towards Avicel (a milled microcrystalline cellulose powder). Both types of enzyme were synthesized in media containing glucose but the activity was influenced by the nature and concentration of the carbon source. The ratio of carboxymethylcellulase activities measured by reducing sugar production and by viscosity decrease was also influenced by the nature of the cellulosic substrate supplied to cultures. The greatest extracellular enzyme production occurred in cultures grown on microcrystalline cellulose powders such as Avicel and Whatman CC3 at concentrations up to 4% (w/v). The greatest degradation rate observed was in cultures supplied with Avicel where 60% of the substrate was degraded in 4 d.

**INTRODUCTION**

The aerobic, mesophilic flexibacterium *Sporocytophaga myxococcoides* has been described as one of the most active cellulolytic micro-organisms known (Berg *et al.*, 1972). Electron microscope studies have shown that it degrades insoluble celluloses by producing cavities at contact sites (Berg *et al.*, 1972b), which indicates that the degradative enzymes are most active in close proximity to the cells. *Sporocytophaga myxococcoides* produces several carboxymethylcellulases, at least two of which act as endoglucanases (Osmundsvag & Goksøyr, 1975). However, these extracellular enzymes have been shown to be inactive on cotton (Osmundsvag & Goksøyr, 1975) and Avicel (Berg *et al.*, 1972b). The aim of the present work was to investigate the spectrum of extracellular cellulases produced by *S. myxococcoides* and the possibility of increasing the yield of these enzymes.

**METHODS**

*Organism and culture media.* *Sporocytophaga myxococcoides* NCIB 8639 was maintained at 4 °C on slopes of basal medium containing 1% (w/v) agar overlaid with strips of Whatman no. 1 filter paper. The basal medium was that of Berg *et al.* (1972a) and was amended with various cellulose substrates or soluble carbohydrates as indicated in the text. Soluble carbohydrates were filter-sterilized and added to the autoclaved basal medium.

*Cellulose substrates.* Avicel SF (technical grade) was obtained from Koch-Light. Bleached wood pulp, prepared by the Stora process, was supplied by Scottish Pulp and Paper Mills, Fort William, Scotland; a full description of this substrate in relation to bacterial cellulolysis is given by Vance *et al.* (1979). Grade 325P uncoated cellophane was supplied by D. J. Parry & Co., 7 Avon Trading Estate, Avonmore Road, London W14. Carboxymethylcellulose type 7L, degree of substitution 0.7, was supplied by Hercules Powder.

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Co., 1 Great Cumberland Place, London. Cellulose powders CC31 (particle size 25 to 30 μm) and CF1 (particle size up to 1000 μm) were obtained from Whatman.

**Bacterial growth.** Growth was estimated by measuring the turbidity of cultures at 525 nm after dilution as necessary. During growth on insoluble substrates, cultures were stored for 1 h at 0 °C before measurement to allow sedimentation of the substrate. This procedure, however, proved unsatisfactory for small particle size substrates such as Whatman CC31 cellulose powder and these cultures were centrifuged for 30 s at 550 g before measuring the turbidity of the supernatant. No attempt was made to estimate growth on the surface of insoluble substrates.

**Enzyme preparations.** Crude preparations of extracellular enzyme were prepared by centrifuging cultures for 30 min at 10000 g and 4 °C. The supernatant was removed and assayed immediately or, in the case of fermenter-grown cultures, stored at -12 °C.

**Assay of activity against Avicel.** The release of soluble carbohydrate from Avicel SF was measured by incubating 1 ml of appropriately diluted crude enzyme with 1 ml of a 1% (w/v) suspension of Avicel SF in distilled water and 2 ml 0·1 M-citrate buffer, pH 5·8 which contained 0·02% (w/v) sodium azide (cf. Mandels & Weber, 1969). Incubation was carried out in stoppered 5 ml volumetric flasks for 24 h at 40 °C in a shaking water bath operating at 120 strokes min⁻¹. Insoluble material was sedimented by centrifuging at 2000 g for 15 min; then 1·9 ml of the supernatant was removed and assayed for carbohydrate by the method of Dubois et al. (1956). Appropriate controls for carbohydrate release from the substrate and that of the enzyme preparation were included. One unit of activity was designated as that amount of enzyme which produced a net increase in absorbance of 0·1 measured at 490 nm in a 1 cm cell.

**Assay of carboxymethylcellulase activity.** The production of reducing sugars from carboxymethylcellulose was determined by incubating 0·5 ml of appropriately diluted crude enzyme with 0·5 ml of a 2% (w/v) carboxymethylcellulose solution in 0·05 M-citrate buffer, pH 5·8. To ensure reproducible initial viscosities the carboxymethylcellulose solution was prepared as a 2% (w/v) solution and stirred overnight at 4 °C on a magnetic stirrer. After mixing the contents of the viscometer, readings were taken of the flow time at intervals over 20 min. The reaction time for each reading was calculated by the method of Fahraeus (1947).

The decrease in viscosity of carboxymethylcellulose was measured in an Ostwald viscometer (size C, Baird & Tatlock) at 30 °C. The reaction mixture consisted of 2 ml of appropriately diluted crude enzyme and 9 ml of 0·5% (w/v) carboxymethylcellulose solution in 0·05 M-citrate buffer, pH 5·8. To ensure reproducible initial viscosities the carboxymethylcellulose solution was prepared as a 2% (w/v) solution and stirred overnight at 4 °C on a magnetic stirrer. After mixing the contents of the viscometer, readings were taken of the flow time at intervals over 20 min. The reaction time for each reading was calculated by the method of Tampion (1965). Activity was expressed in terms of mol 1,4-β-glucosidic bonds broken s⁻¹ by the use of an equation allowing the insertion of specific viscosity measurements. The equation was essentially that of Almin & Eriksson (1975) but α, an empirical constant introduced by Almin & Eriksson (1967), was set equal to unity. The Baker equation (Almin & Eriksson, 1967) adequately described the relation between specific viscosity and substrate concentration when an experimentally derived value of α = 3·0 was used. Values of x = 0·86 and $C_m = 1·33 \times 10^{-2}$ 1 g⁻¹, the exponent and constant in the modified Staudinger equation (Almin & Eriksson, 1967), were taken from Almin & Eriksson (1968) and Hulme (1971), respectively. One unit of activity was the amount of enzyme which breaks 1 nmol 1,4-β-glucosidic bonds s⁻¹.

**Effect of nature and concentration of cellulose substrate.** Shake flask cultures were grown in 250 ml Erlenmeyer flasks which contained 100 ml basal medium amended with 1 g cellulose substrate as indicated in the text. Substrates supplied in sheet form, i.e. filter paper, pulp fibre and cellophane, were cut into approximately 1 cm² pieces. Cultures were inoculated with 5 ml of a 4 d shake flask culture of S. myxococcusoides grown in basal medium amended with glucose (0·1%, w/v). Three replicates of each culture were incubated at 30 °C on an orbital shaker operating at 100 rev. min⁻¹. Samples were withdrawn after 4 d and assayed for extracellular carboxymethylcellulase. Residual cellulose in the cultures was determined by the gravimetric method of Fahraeus (1947).

**Cellulose fermentation profile.** Cultures were grown in a 10 l stirred tank fermenter (L.H. Engineering, Stoke Poges, Bucks.) with a working volume of 7 l. Temperature was maintained at 30 °C and the contents were sparged with air at a rate of 2 l min⁻¹. Mixing was achieved with two vaned discs revolving at 200 rev. min⁻¹. Basal medium was amended with various carbon sources as indicated in the text. In an attempt to reduce foaming, Tween 80 (0·1%, v/v) was added to the culture grown on glucose. However, more satisfactory control of foaming was achieved by semi-continuous addition of Dow Corning 525 emulsion at a rate of 0·1 ml h⁻¹. Dissolved oxygen was measured with a type E1-1-04 electrode (L.H. Engineering) and pH was measured with a type CW/EXT/710 combination electrode (Russel pH, Auchtermuchty, Fife). Culture samples were removed at intervals and assayed for extracellular carboxymethylcellulase and activity on Avicel. Reducing sugars in the culture supernatant were estimated by the method of Nelson (1944).
**Table 1. Degradation of cellulose substrates and production of cell-free carboxymethylcellulase in 4 d shake flask cultures of S. myxococcoides**

<table>
<thead>
<tr>
<th>Substrate (1 %, w/v)</th>
<th>Degradation (%)</th>
<th>DNS activity (units ml⁻¹)</th>
<th>Viscometric activity (units ml⁻³)</th>
<th>Ratio of viscometric to DNS activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avicel SF</td>
<td>60</td>
<td>24</td>
<td>62.7</td>
<td>2:6:1</td>
</tr>
<tr>
<td>Whatman CC31</td>
<td>40</td>
<td>17:2</td>
<td>31:8</td>
<td>1:9:1</td>
</tr>
<tr>
<td>Whatman CF1</td>
<td>33</td>
<td>5:3</td>
<td>28:5</td>
<td>5:4:1</td>
</tr>
<tr>
<td>Whatman filter paper no. 1</td>
<td>31</td>
<td>7:3</td>
<td>40:7</td>
<td>5:5:1</td>
</tr>
<tr>
<td>Stora wood pulp</td>
<td>19</td>
<td>7:0</td>
<td>8:7</td>
<td>1:2:1</td>
</tr>
<tr>
<td>Cellophane 325P</td>
<td>14</td>
<td>5:3</td>
<td>6:6</td>
<td>1:2:1</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

*Sporocytophaga myxococcoides* utilized a variety of cellulose substrates when these were supplied as carbon sources in shake flask cultures. Of the six substrates tested, Avicel SF (a milled substrate) was degraded fastest (Table 1). The degradation of 60% of the supplied Avicel, observed in the present work, is very similar to the rate reported by Berg et al. (1972a) for cultures of *Cellvibrio fulvus*. In the same study these workers found only a 10% decomposition of a non-specified Whatman cellulose powder over 4 d incubation whilst some 65% of sulphite pulp fibres was degraded over the same period. Kaufmann et al. (1976) also observed that Avicel was degraded more rapidly than Whatman CF1 cellulose powder when supplied to a cellulolytic bacterium isolated from soil. The results obtained for *S. myxococcoides* indicate that milled microcrystalline cellulose substrates such as Avicel are degraded faster than other microcrystalline substrates such as Whatman cellulose powder and more amorphous cellulosics such as wood pulp, especially if the latter are supplied in a form which presents a reduced area of contact with the bacteria. The topography of the substrate particles is likely to be important in determining its rate of utilization by *S. myxococcoides* since the electron microscope studies of Berg et al. (1972b) indicate that this bacterium colonizes all of the available surface of the substrate including the lumen of hollow fibres.

In the present work the cellulolytic activity is described as extracellular since it was assayed in the cell-free culture supernatants. There is difficulty, however, in interpreting the physiological role of these enzymes. It is not possible to say if such enzymes have been actively secreted by the bacteria or if they are products of cell lysis, which is known to occur even during exponential growth of bacterial cultures (v. Hofsten, 1975). The extracellular carboxymethylcellulase activity produced by *S. myxococcoides* was found to vary with the nature of the carbon source supplied to cultures (Table 1), an observation also made on carboxymethylcellulase production by *Cellvibrio fulvus* (Berg et al., 1972a) and *Pseudomonas fluorescens* (Suzuki et al., 1969). The greatest extracellular carboxymethylcellulase activity produced by *S. myxococcoides* was in cultures supplied with Avicel as the sole carbon source. Suzuki et al. (1969) made the same observation when Avicel was compared with 15 other carbon sources including carboxymethylcellulose, mono-, di- and oligosaccharides. We have also found that the nature of the cellulose supplied to *S. myxococcoides* appeared to influence the ratio of carboxymethylcellulase activities recovered from the cell-free medium (Table 1). The viscometric technique may be regarded as an assay for endo-1,4-β-glucanase activity (EC 3.2.1.4) whilst the production of reducing sugars reflects equally the capability of exo-1,4-β-glucanase (EC 3.2.1.74) and endoglucanase to hydrolyse carboxymethylcellulose. A randomly acting endoglucanase would be expected to produce a rapid decrease in viscosity in relation to the number of reducing groups (i.e. a high ratio of viscometric to reducing sugar activity) and a less randomly acting endoglucanase or an exoglucanase would be expected to produce a lower ratio (Wood & McCrae, 1978). The
highest ratios of viscometric to reducing sugar activity were found in cultures grown on Whatman CF1 cellulose powder and Whatman no. 1 filter paper. Interestingly, the ratio was lower when CC31 cellulose powder was used as a substrate. The CC31 powder is treated with hydrochloric acid during manufacture and hence forms a more microcrystalline substrate than the CF1. Comparisons between the two celluloses are, however, complicated by their different particle sizes. Although the higher ratio observed with the CF1 cellulose argues for a change in the spectrum of enzyme production with endoglucanase becoming dominant, the results may also be explained in terms of the enzyme binding properties of cellulose substrates. Halliwell & Griffin (1978) have reported, for example, that components of the cellulase complex of Trichoderma koningii are adsorbed to microcrystalline cellulose with varying degrees of tenacity and some components may be permanently adsorbed by other celluloses.

The extracellular carboxymethylcellulase and activity against Avicel in S. myxococcoides cultures could be increased by including higher concentrations of cellulose, at least up to 4% (w/v), in the medium. When Whatman CC31 cellulose powder was supplied to cultures of S. myxococcoides at 0·1, 1, 2 and 3% (w/v) the cell-free carboxymethylcellulase detected using the DNS assay, after 4 d growth, was 9, 77, 82 and 98%, respectively, of the activity found in a culture supplied with 4% (w/v) cellulose. Sternberg & Dorval (1979) have shown that the greatest yield of extracellular cellulase from Trichoderma reesei was obtained with 8% (w/v) cellulose. The use of media with a high cellulose content appears to be desirable when greater yields of cellulase are required. However, this effect does not appear to be universal since Szajer & Targonski (1977) report that cellulose concentrations greater than 0·75 to 1·2% (w/v) inhibited the production of carboxymethylcellulase by Fusarium sp. Other disadvantages of high cellulose content media are that they are difficult to aerate and agitate efficiently in conventional fermenters.

Enzyme production in a stirred tank fermenter during growth in basal medium amended with 0·1% (w/v) glucose, 0·1% (w/v) Whatman CF1 cellulose powder or 2·0% (w/v) Whatman CC31 cellulose powder is shown in Fig. 1(a), (b) and (c), respectively. Sporocytophaga myxococcoides grew faster on 0·1% glucose than on 0·1% CF1 cellulose powder with maximum growth rates of 9·1 × 10⁻⁸ $A_{562}$ units h⁻¹ and 6·2 × 10⁻⁸ $A_{562}$ units h⁻¹, respectively. However, the highest growth rate, 4·9 × 10⁻⁷ $A_{562}$ units h⁻¹, was observed in the culture supplied with 2% CC31 cellulose. The maximum enzyme activity measured by the three assays was also greatest in the 2% CC31 cellulose culture and in each case was approximately 25-fold greater than the maximum activities in the 0·1% CF1 cellulose culture. Activity against Avicel was only assayed after 90 h growth in the culture supplied with glucose and at this time the activity was of the same order as the maximum activities in the 0·1% CF1 cellulose culture. At the time of this assay the glucose concentration of the medium was 0·1 mg ml⁻¹. Essentially the carboxymethylcellulase activities in the 0·1% glucose culture were greater than in the 0·1% cellulose culture, the maximum activity measured by the reducing sugar assay being 50% greater and that measured by the viscometric assay being some 400% greater. The increased carboxymethylcellulase activity in the 0·1% glucose culture may be due to the action of the surfactant added to control foaming or to the tendency of cellulase to bind to the residual cellulose in cultures. The carboxymethylcellulase activity and activity against Avicel found in the glucose culture may have arisen because of the presence of filter paper and its degradation products in the inoculum rather than being a result of constitutive enzyme synthesis. In all the fermenter-grown batch cultures the

Fig. 1. Growth and extracellular enzyme production of S. myxococcoides in basal medium amended with (a) 0·1% (w/v) glucose, (b) 0·1% (w/v) Whatman CF1 cellulose powder or (c) 2% (w/v) Whatman CC31 cellulose powder: $\square$, growth ($A_{562}$); $\bullet$, pH; $\square$, dissolved oxygen; $\blacklozenge$, carboxymethylcellulase, by reducing sugar production; $\triangle$, carboxymethylcellulase, by viscometry; $\triangleleft$, activity on Avicel; $\triangledown$, residual glucose.
Cellulase production by Sporocytophaga

For legend see opposite.
maximum enzyme activity was found during the late-exponential phase of growth and the detectable activity decreased as the culture continued into the stationary phase. The period of maximum growth and enzyme production was characterized by a depression of the medium pH and dissolved oxygen concentration (Fig. 1c).

Contrary to the findings of Berg et al. (1972b), we have demonstrated that S. myxococoides produces an extracellular enzyme which degrades Avicel and is detectable by measuring the soluble carbohydrate released. Although Berg et al. (1972b) provide no experimental detail it is possible that assaying for soluble carbohydrate production may be a more sensitive way of detecting cellulose hydrolysis than assaying for reducing sugars, particularly if the hydrolysis products were predominantly oligosaccharides. Chang & Thayer (1977) have also described a species of Cytophaga which did not produce extracellular cellulase or carboxymethylcellulase regardless of culture conditions. In the present work Tween 80 and Dow Corning 525 emulsion were used to control foaming in fermenter cultures and there is evidence that such surfactants increase the yield of extracellular cellulases, presumably by increasing cell permeability (Reese & Maguire, 1969; Mandels & Weber, 1969). However, the inclusion of surfactants did not cause the appearance of the extracellular activity towards Avicel since this was detected in the absence of anti-foam (Fig. 1b).

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REFERENCES


