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Characterization of the cellulase complex from *Cellulomonas* grown on bagasse pith

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Summary. The effect of physico-chemical parameters on the cellulolytic activity of *Cellulomonas* sp. IIbc grown on sugarcane bagasse pith was investigated, and the optimum ranges for enzyme activity were established. The cellulases were more stable when incubated at the optimum growth temperature (32° C) than under optimum activity conditions (45° C for β-glucosidases and 50° C for CMC- and FP-cellulases). The β-glucosidases were the thermostability-limiting enzymes of the complex. Two types of endoglucanases could be recognized according to their adsorption properties on bagasse: one weakly-bound and one tightly-bound type, the latter constituting approximately 73% of the extracellular endoglucanases at exponential growth phase. Four forms active on filter paper and three active on CMC were obtained by HPLC separation of the extracellular fraction of the culture at stationary phase.

Introduction

Cellulolytic bacteria represent an important perspective for the production of single cell protein from lignocellulosic materials (Dunlap 1969; Enríquez 1981). The study of the cellulolytic enzymes produced by the bacteria during this process is of considerable importance for its better understanding and application. Several studies have been carried out on the characterization of bacterial cellulases in different species (Bevers 1976; Chang and Thayer 1977; Haggett et al. 1979). However, the characteristics of the enzymes produced during the growth on lignocellulosic substrates have not been extensively investigated. The objective of this work has been the study of some of the most important characteristics and properties of the cellulolytic enzymes produced by *Cellulomonas* sp. IIbc during its growth on sugarcane bagasse pith.

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Abbreviations: CMC, carboxymethylcellulose; FP, filter paper

Materials and methods

Strain and cultivation conditions. Cellulomonas sp. IIbc (Enríquez 1981) was cultivated in fermentors in the conditions and medium previously described (Rodríguez and Volfová 1984). Alkali pretreated sugarcane bagasse pith (Dunlap 1969) was used as the carbon source (10 g/l).

Effect of physico-chemical parameters on enzyme activity. The effect of pH, temperature and incubation time on cellulolytic activity was evaluated in a total culture sample taken at exponential growth phase. For determination of activity against CMC, 0.5 ml of the sample was mixed with 2.5 ml of 1.25% CMC solution (BDH, degree of substitution 0.7–0.8), and 2 ml of buffer. For activity on FP, 0.5 ml of the sample was incubated with 1 ml of buffer and 50 mg of Whatman No. 1 filter paper. Reducing sugars after incubation were determined by the Somogyi-Nelson method (Nelson 1944; Somogyi 1952). Aryl-β-glucosidase activity was measured as the amount of 4-para-nitrophenol released from 1 ml of 0.0136 M 4-nitrophenyl-β-glucoside, after incubation with 0.5 ml of enzyme and 0.5 ml of buffer. Different temperatures, pH and incubation times were assayed in the three cases. McIlvaine buffer was used for pH 3.5 to 5.0, and phosphate buffer for pH 5.5 to 8.0. The activity is expressed in IU (μmoles of products per minutes), per ml. The experiments were run in duplicate.

The endoglucanase activity was determined by the decrease in viscosity of a 0.5% CMC solution, according to Ravinovitch et al. (1977).

Stability. A filtered cell suspension was incubated at different conditions of pH and temperature. During the incubation, samples were taken at different times and evaluated for CMC-, FP- and β-glucosidase activity.

Adsorption of cellulases on bagasse. The supernatant liquor from a culture at stationary phase (pH 6.5) was analysed. A total of 0.5 IU of endoglucanase activity was passed through a column containing 8.1 g of ball milled bagasse pith (particle size 125 μ). The liquid eluted from the column was collected in fractions and the endoglucanase activity of each fraction was determined. After all the enzyme-containing liquid had passed through the column, this was finally washed with phosphate buffer (pH 6.5), and the activity determined in this effluent too. The total eluted activity as a percentage of the initial activity injected into the columm was regarded as the percentage of weakly adsorbed enzymes.

High performance liquid chromatography (HPLC). The supernatant liquor from a sample from stationary phase was used for the experiment. The chromatographic procedure was as that described by Hostomská and Mikeš (1983), employing the ion-exchange derivative of Spheron DEAE-1000 (Spheron diethylaminoethyl-1000, Lachema, Czechoslovakia). Chromatography proceeded on a glass column (0.2 × 0.008 m) at a pressure of 0.5 MPa, with a mean flow of 0.2 ml/min. Fractions were collected at 60-sec intervals and evaluated for cellulolytic activity.

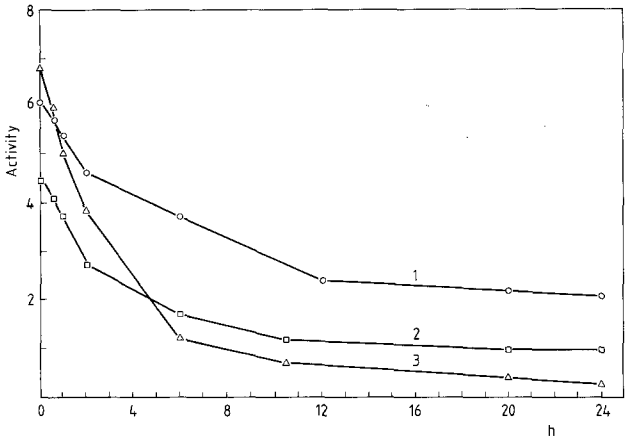

Fig. 2. Stability of the cellulolytic enzymes (cell suspension sample) incubated at the optimum activity conditions. *1:* CMC-activity (IU/ml × 10^2); *2:* FP-activity (IU/ml × 10^2); *3:* β-glucosidase activity (IU/ml × 20)

Results and discussion

The effect of pH and temperature on the cellulolytic activity of the total culture sample is shown in Fig. 1.

Optimum conditions for the enzymatic activity were as follows: for CMC-activity pH 6.5, temperature 50° C, incubation time 0.5 h; for FP-activity pH 7.0, 50° C and 1 h; for β-glucosidase activity pH 7.0, 45° C and 1 h. As can be seen, the optimum temperature for the cellulolytic activity of this strain is different from the optimum growth temperature (32° C, Enríquez 1981), although the optimum pH values coincide for both cell growth and enzyme activity.

It was observed that there was no linearity in the release of the reaction products with time, between 0.5 and 24 h of incubation. Similar results were found by Bevers (1976) for a bacterial isolate, and by Mandels et al. (1976) for *Trichoderma viride.*

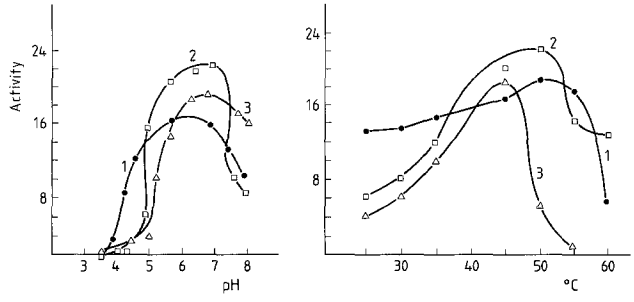

Fig. 1. Effect of pH and temperature on the cellulolytic activity of a total culture sample. *1:* CMC-activity (IU/ml × 10^2); *2:* FP-activity (IU/ml × 8 × 10^2); *3:* β-glucosidase activity (IU/ml × 40)

Stability

Figure 2 shows the stability of the enzymes incubated at optimum conditions of activity.

After 24 h of incubation, the loss in activity was 65% for CMC-cellulases, 77% for FP-cellulases and 90% for β-glucosidases, with respect to activity at time zero. Since a crude extract was used in this study, these are properties of the mixture of enzymes.

The stability of a similar sample, incubated at 32° C and 2 different pH is shown in Fig. 3. The cellulases were more stable at the optimum growth conditions (32° C and pH 6.5) than at the optimum activity conditions (Fig. 1). At the optimum growth parameters the total loss in activity was 28% for CMC-cellulases, 31% for FP-cellulases and 19% for β-glucosidases. At this lower temperature, the β-glucosidases become much more stable. This drastic loss of stability with temperature increase for the β-glucosidases has also been reported by Fähnrich and Irrgang (1982) for *Chaetomium cellulolyticum.* Besides this, the stability of FP-cellulases and β-glucosidases was readily influenced by pH, while the CMC-cellulases showed similar stability at both incubation pH.

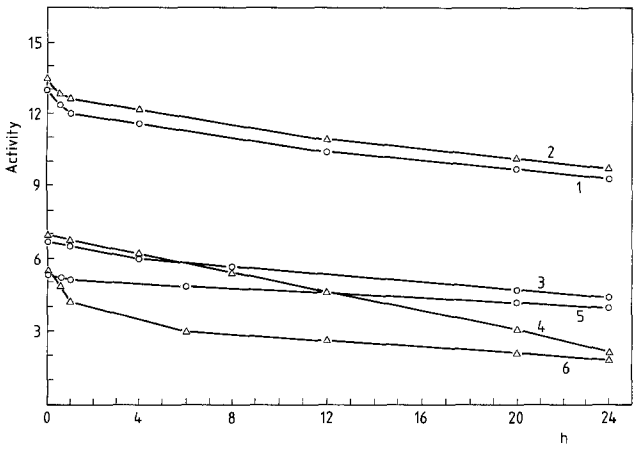

Fig. 3. Stability of the cellulolytic enzymes (cell suspension sample) incubated at 32° C. *1* and *2:* CMC-activity at pH 6.5 and 4.8, respectively (IU/ml × 10^2); *3* and *4:* FP-activity at pH 6.5 and 4.8, respectively (IU/ml × 10^3); *5* and *6:* β-glucosidase activity at pH 6.5 and 4.8, respectively (IU/ml × 20)

Chromatographic fractionation of the enzymes

The HPLC separation of the supernatant sample from a culture at stationary phase is shown in Fig. 4.

Four forms active on FP were obtained. Three of them showed a low CMC-activity, while in the other one, a high CMC-activity is also present. Three peaks are distinguishable for forms active on CMC. The imperfect separation, resulting in peaks with both CMC- and FP-activity, has also been reported by other authors (Fliess and Schüger 1983; Hostomská and Mikeš 1983). This fact seems to be due to the unespecificity of the enzyme assays, that is to say, to the fact that a single cellulase would be able to produce reducing sugars from both substrates, CMC and FP, although certainly with a different efficiency.

From the pattern obtained it can be suggested that at least six minor cellulase components are present in these *Cellulomonas* cultures on bagasse pith at stationary phase, five of them showing different cross-specificity toward CMC and FP as substrates. Since at this stage of growth almost all CMC- and FP-cellulases are released to the medium (Rodríguez and Volfová 1984), this pattern would represent an important part of the whole cellulase complex produced by this strain, although the possibility of some fractionation of original components cannot be discarded, and in future conclusive results will depend on a detailed analysis of the purified enzymes.

No β-glucosidase activity was detected in the supernatant fraction of the crude extract used in the HPLC separation. This is in agreement with previous results about the cell-bound nature of the β-glucosidases from this strain (Rodríguez and Volfová 1984).

Adsorption

The adsorption characteristics of fungal cellulases on cellulose have been studied by several authors (Klyosov 1980; Reese 1982). However, there has been little investigated of bacterial cellulases and their adsorption on lignocellulosic substrates. Figure 5 shows the results of the adsorption experiment.

The total endoglucanase activity eluted from the column was 0.136 IU, which represents the

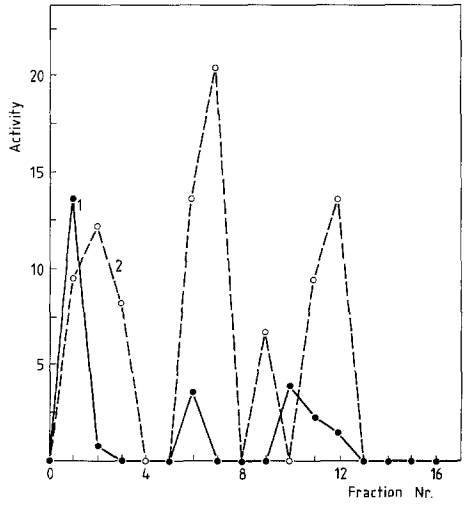

Fig. 4. HPLC of free enzymes from stationary phase. *1:* CMC-activity (IU/ml × 10^3); *2:* FP-activity (IU/ml × 10^3)

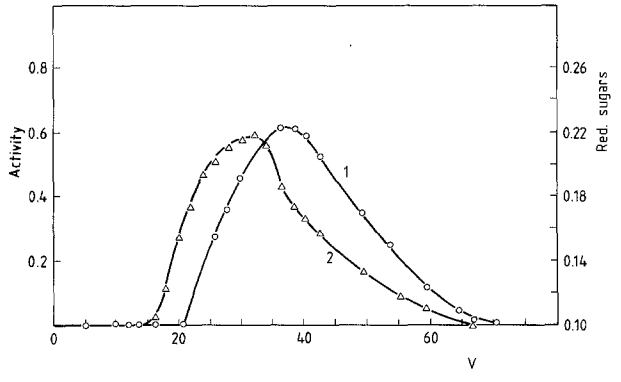

Fig. 5. Adsorption of endoglucanases on a bagasse column. *1:* Activity in the eluted samples (IU/ml × 10^2, viscosimetric measurement); *2:* Reducing sugars in the eluted samples (mg/ml); *V:* Total eluted volume (ml)

27% of the injected activity. This suggests that a maximum of 73% of the endoglucanases remained bound to the bagasse, even after buffer washing. Taking into account that some inactivation of the enzymes could also take place, this would represent the maximum percentage of "bound enzymes". This means that at least two types of endoglucanases, differing in their adsorption characteristics, are present in the supernatant fraction of these cultures: one "weakly bound type" and one "tightly bound type", with predominance of the latter. This agrees with the general observation on the necessity for close contact between cellulases and their substrates for the degradation of cellulose.

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