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Short communication

Formation and localization of cellulases in *Cellulomonas* culture on bagasse

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Summary. The cellulolytic enzyme complex was studied during the diauxic growth of Cellulomonas sp.IIbc on alkali-pretreated sugar cane bagasse pith. In the first growth phase only a low cell-bound aryl-β-glucosidase activity was detected. Formation of extracellular and bound (cell-, bagasse-) CM- and FP-cellulases occurred later, i.e. at the beginning and during the second growth phase. The levels of all cellulolytic enzymes, mainly bound ones, increased with the growth of cells. At the end of the linear growth phase almost all bound cellulolytic enzymes, except for cell-bound aryl-β-glucosidase, are released to the medium as an extracellular complex. A considerable level of the intracellular aryl-β-glucosidase activity is still present at the end of the fermentation.

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Introduction

The growing interest in microbial cellulases in recent years arises from their potentional use for the industrial saccharification of cellulosic substrates and for SCP production. In the latter direction, numerous studies have been undertaken on the cultivation of cellulolytic bacteria on pretreated lignocellulosic wastes for protein production (Hanz and Srinivassan 1968; Dunlap 1969; Enriquez 1978; 1981). In countries with a large sugar cane industry, the industrial wastes known as bagasse, can constitute particularly suitable substrates for this process. Before a practical application of the process, knowledge of the enzymatic mechanism involved and its behaviour during bacterial growth on the cellulosic substrate is required. It was the aim of the present work to characterize precisely the formation and localization of the cellulolytic enzyme complex during the growth of Cellulomonas on pretreated sugar cane bagasse pith in the SCP production system.

Material and methods

Strain. Cellulomonas sp IIbc (Enriquez 1978).

Media. The strain was maintained on CMC-agar and cultivated on optimized cultivation medium (Rodriguez et al. 1983) of the following composition (g/l): KH_2PO_4 0.6; NH_4Cl 2.0; NaCl 0.25; $MgSO_4.7H_2O$ 0.25; Thiamine 0.01; alkaline pretreated bagasse (Dunlap 1969) pith 10.

Cultivation conditions. Cultivation was in a 3-liter fermentor (impeller speed 10 Hz; aeration rate 1.5 l/min; temperature 32°C; constant pH 6.5, regulated automatically by addition of 10% sodium hydroxide). Inoculum (10 % (v/v)) was prepared in shaken flasks, centrifuged and the sediment was resuspended in the same volume of the sterile medium.

Assessment of enzyme activities. Enzyme activities were determined under the optimum assay conditions (Rodriguez 1983).

Carboxymethyl-(CM-)cellulase activity was assayed as the amount of reducing sugars released from carboxymethylcellulose after a 30-min incubation at 50°C and pH 6.5. Filter paper-(FP-)cellulase activity was determined as the amount of reducing sugars released from a strip of Whatman No.1 filter paper after a 60-min incubation at 50°C and pH 7.0.

Aryl-β-Glucosidase activity was assayed according to the modified method of Okada et al. (1968) as the amount of 4-nitrophenol released from 4-nitrophenyl-β-glucoside after a 60-min incubation at 45°C and pH 7.0.

The extracellular activity was determined in the supernatant after centrifugation of the culture at 15 000xg for 10 min.

The cell bound activity was calculated from the difference between the activity of intact cell suspension and the activity of the supernatant. For the determination of the bagasse bound activity, the sample was filtered through a sintered glass filter No.1 porosity and the bagasse was resuspended in the same volume of the medium. The intracellular enzyme activity was determined in a cell free extract from washed cells. The suspension of bacteria and residual bagasse were filtered through sintered glass filter No.1 porosity (in order to remove bagasse) and the filtrate was centrifuged at 15 000xg for 15 min. The sediment was washed and resuspended in phosphate buffer pH 7.0 (0.6 g/ml), disrupted in a Braun disintegrator and centrifuged at 26 000xg for 30 min. The supernatant was assayed for the intracellular activity. The sediment was made up to the initial volume with buffer and assayed for the cell debris bound activity. Reducing sugars were determined by the Somogyi-Nelson method (Somogyi 1952).

The growth of cells was followed turbidimetrically after filtration of the sample through the sintered glass filter No.1 porosity at 600 nm.

The extracellular proteins were determined according to Lowry et al. (1951).

Results and discussion

The diauxic growth of Cellulomonas sp. IIbc was detected in most of the cultures on alkali-pretreated sugar cane bagasse pith. The prediction (Enriquez 1981) that this strain grows first on the residual hemicellulose and, after an adaptation period, on cellulose of the pretreated bagasse pith was fully confirmed by our detailed studies of the cellulolytic complex in the Cellulomonas culture under constant cultivation conditions. At the beginning of cell growth and during the first growth phase, no cellulases (FP- and CM-) were detected in the system. At this time only low levels of cell-bound aryl-β-glucosidase, introduced probably with the original inoculum, were detected (Fig. 1). During this phase, the amount of reducing sugars in the medium rapidly decreased (Fig. 2). As only solid sediment of the culture served as inoculum, the content of free reducing sugars in the medium at the beginning of cell growth should increase due to the rapid degradation of bagasse hemicellulose. The high xylanase activity and decreasing hemicellulose content in the system (Rodriguez 1983) supported this as-

sumption. The CM-cellulase activity is derepressed at the end of the lag between the first and second growth

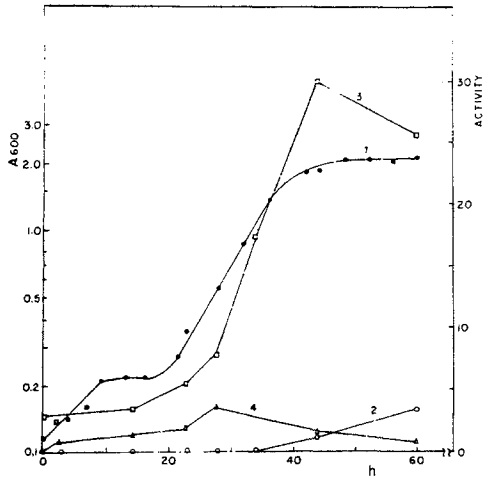

Fig. 1. The levels of aryl-β-glucosidase activities (IU/ml x 10^2) in different culture fractions during the growth of Cellulomonas sp. IIbc on pretreated bagasse pith. 1, cell growth (absorbance at 600 nm); 2, extracellular enzyme activity; 3, cell-bound enzyme activity; 4, bagasse-bound enzyme activity

phases; simultaneously the cell-bound, extracellular and bagasse-bound enzymes become active (Fig. 3). The levels of enzyme activities then increase with increasing cell growth and increasing level of extracellular proteins (Fig. 2). Similarly, but somewhat later, FP-cellulase becomes active in the system, first as the cell-bound enzyme but is later also excreted to the medium and binds to the bagasse particles (Fig. 4).

During active cell growth on bagasse pith, CM- and FP-cellulases, unlike aryl-β-glucosidase, exist as extracellular and bound enzymes. During the

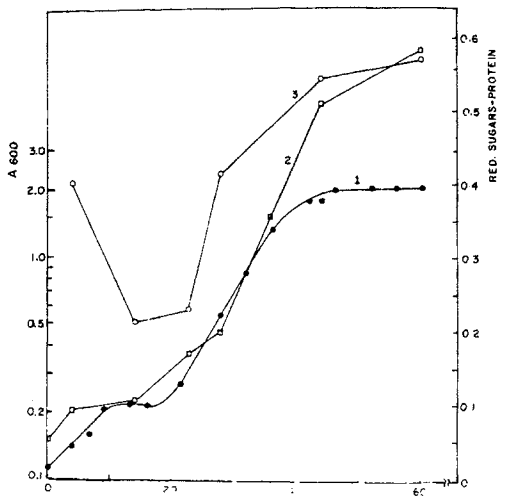

Fig. 2. The production of extracellular proteins and reducing sugars during the growth of Cellulomonas sp. IIbc on pretreated bagasse pigh. 1, cell growth (absorbance at 600 nm); 2, extracellular proteins (mg/ml); 3, free reducing sugars (mg/ml x 3.4)

whole fermentation aryl-β-glucosidase remains mainly cell-bound (Fig. 1) exhibiting a high intracellular activity until the end of the fermentation (Table 1). The low level of bagasse--bound aryl-β-glucosidase (Fig. 1) is probably associated with its other functions and originates from the bacterial cells attached to the bagasse particles. A significant and rapid decrease in the level of cell- and bagasse-bound FP- and CM-cellulase during the second phase of the active cell growth on bagasse pith (Figs. 3 and 4) and the low level of intracellular FP-cellulase at the end of fermentation (Table 1) are not due to a limitation by bagasse. It is possible that a repression of cellulase synthesis by reducing sugars (Fig. 2) might

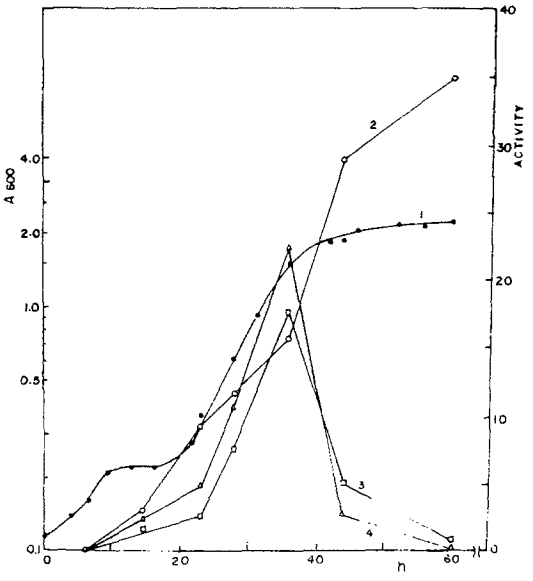

Fig. 3. The levels of CM-cellulase activities (IU/ml x 10^2) in different culture fractions during the growth of Cellulomonas sp. IIbc on pretreated bagasse pith. 1, cell growth (absorbance at 600 nm); 2, extracellular enzyme activity; 3, cell-bound enzyme activity; 4, bagasse-bound enzyme activity

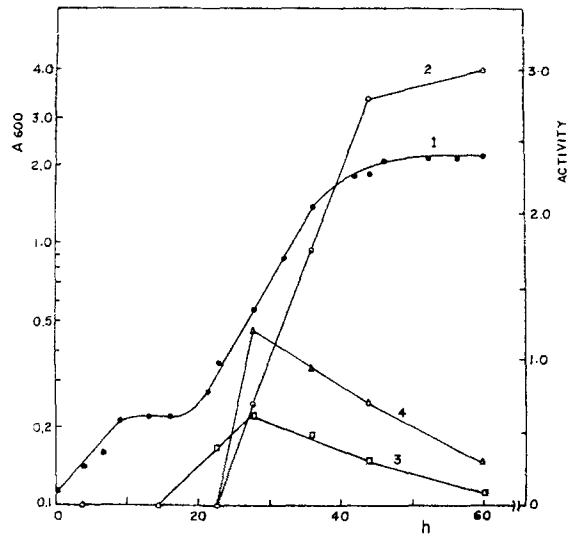

Fig. 4. The levels of FP-cellulase activities (IU/ml x 10^2) in different culture fractions during the growth of Cellulomonas sp. IIbc on pretreated bagasse pith. 1, cell growth (absorbance at 600 nm); 2, extracellular enzyme activity; 3, cell-bound enzyme activity; 4, bagasse-bound enzyme activity

Table 1. Intracellular enzyme activity of Cellulomonas sp. IIbc grown on pretreated bagasse pith

Growth Phase	Fraction[a]	Enzyme activity (IU/ml x 10)		
		FPA	CMCA	β-GA
Exponentional	Intracellular	0	1.51	0.40
	Cell debris	0	1.63	0.52
Early stationary	Intracellular	0.24	0.65	-[b]
	Cell debris	0.03	0.76	-[b]
Late stationary	Intracellular	0.09	0.68	1.70
	Cell debris	0.01	0.40	0.47

[a] Obtained as described in Methods
[b] Not determined

be involved. Relatively high amounts of non-degraded bagasse pith and high levels of the active extracellular cellulolytic complex in the system at the end of fermentation (Figs. 1, 3 and 4) also indicate that residual lignin might probably interfere with the availability of the residual cellulose to the bacterial enzymes in the complex of bagasse structure.

It follows from the above results that the synthesis of cellulases in Cellulomonas is inducible and that the cell-bound aryl-β-glucosidase could play a role in the formation of a natural cellulase inducer. Compared with fungal cellulases, the enzymes refer-

red to here are mainly bound i.e. bound to the cells and to bagasse particles. Their extracellular forms predominate only at the end of the fermentation.

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