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SHORT COMMUNICATION

Cellulolytic Enzyme System of *Acetivibrio cellulolyticus*, a Newly Isolated Anaerobe

By A. W. KHAN

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

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Crude enzyme preparations from *Acetivibrio cellulolyticus* converted ball-milled pulp, cotton batting, filter and tissue paper, a microcrystalline cellulose, carboxymethylcellulose, cellobiose and xylan to reducing sugars. The preparations showed maximum activity between pH 5 and 6 and at a temperature between 37 and 50 °C, depending on the substrate used. The enzyme activity was fairly stable at 2 °C for 4 weeks. The saccharifying ability of the preparation was comparable to that of commercially available cellulase preparations from *Aspergillus niger* and *Trichoderma viride*.

INTRODUCTION

Recovery and re-use of waste cellulose is being studied extensively as a way to alleviate energy and food shortages (Ghose, 1977). One approach is enzymic hydrolysis to convert cellulosic materials into glucose. This involves at least four major enzyme complexes: endoglucanase, exoglucanase, cellobiosylhydrolase and cellobiase (Ghose & Ghose, 1979). Endoglucanase randomly splits the cellulose polymer into smaller components, whilst exoglucanase removes one glucose unit at a time from the non-reducing end of the split molecule (Ghose & Ghose, 1979). The action of cellobiosylhydrolase is similar to exoglucanase except that it removes cellobiose units from the chain (Lee *et al.*, 1978). Cellobiase catalyses the hydrolysis of cellobiose to glucose (Ghose & Bisaria, 1979). In addition to these four enzymes, xylanase is also required for the hydrolysis of natural cellulose. Cellulase from *Trichoderma* sp. is deficient in xylanase and its activity is increased when mixed with xylanase (Ghose *et al.*, 1976).

This paper describes a cellulolytic enzyme system of *Acetivibrio cellulolyticus*, a newly isolated anaerobe (Khan *et al.*, 1980; Patel *et al.*, 1980) capable of degrading a variety of cellulosic materials (Khan *et al.*, 1980). The ability of crude enzyme preparations of this organism to convert cellulosic materials to reducing sugars was compared with commercially available cellulase preparations.

METHODS

Organism and growth. Acetivibrio cellulolyticus (strain CD2, National Research Council no. 2248) was isolated and characterized as described elsewhere (Khan *et al.*, 1980; Patel *et al.*, 1980); it is a fastidiously anaerobic, Gram-negative, rod-shaped bacterium and degrades cellulose and cellobiose to acetic acid, H_2 and CO_2 but does not grow on glucose. A synthetic salt/vitamin medium (Khan *et al.*, 1979) was supplemented with cellulose (2 g l^{-1}, CF-11; Whatman), a reducing agent (1·25 mM-cysteine.HCl and 1·25 mM-Na_2S)

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Table 1. *Cellulolytic activities of Acetivibrio cellulolyticus and commercially available enzyme preparations from Aspergillus niger and Trichoderma viride*

	Cellulolytic activity [μg glucose released (mg protein)$^{-1}$ min^{-1}]			
	*A. cellulolyticus**			
Substrate	Culture supernatant	Cell-free extract	*Aspergillus niger*	*Trichoderma viride*
Absorbent cotton batting	0·4	1·0	0·1	0·5
Ball-milled pulp	1·1	2·8	1·7	0·5
Carboxymethylcellulose	163·0	ND	109·0	78·0
D-(+)-Cellobiose	7·5	56·6	ND	ND
Facial tissue paper	0·5	0·8	1·0	0·1
Filter paper	1·0	2·6	2·2	0·5
Microcrystalline cellulose	2·8	4·1	2·3	0·8
Xylan	66·0	ND	49·0	52·0

ND, Not determined. * 4 to 5 d culture.

and resazurin solution (1 mg l^{-1}) as a redox potential (E_h) indicator. The pH of the medium was adjusted to 7·2, and it was then prereduced by the Hungate technique (Hungate, 1950), dispensed in 50 ml portions in 160 ml serum vials (Miller & Wolin, 1974) under N_2/CO_2 (80:20, v/v) and autoclaved at 103 kPa (15 lbf in^{-2}) for 15 min. One ml of a 3 d culture was used as inoculum. All cultures were incubated for 4 to 5 d at 37 °C with shaking.

Measurement of cellulolytic activity. Cultures were harvested by centrifuging at 12000 *g* for 30 min and the supernatant obtained was used for measuring the exogenous cellulolytic activity. Total cellulolytic activity was measured in cell-free extracts prepared by disrupting the organism by two passages through a French pressure cell at 83 to 110 MPa; the remaining whole organisms and cell debris were removed by centrifuging at 12000 *g* for 15 min.

The enzyme assay mixture contained, in 4 ml, 400 μmol acetate buffer, pH 5·0 or KH_2PO_4/K_2HPO_4 buffer, pH 6·0, 20 mg substrate and 2·0 ml cell-free extract or culture supernatant. The mixtures were incubated at 37 °C with shaking for 1 h, except when cellobiose, carboxymethylcellulose or xylan were the substrates, in which case the assay mixtures were incubated at 50 °C for 30 min. Immediately after incubation the assay mixtures were quickly filtered using a Swinnex-filter holder (Millipore) and a syringe.

In most cases, reducing sugars were assayed colorimetrically using the *o*-toluidine reagent (Feteris, 1965) With carboxymethylcellulose and xylan, the reducing sugars were assayed using dinitrosalicylic acid (Miller, 1959); while for D-(+)-cellobiose, glucose was assayed by the use of the glucostat enzyme method (Raabo & Terkildsen, 1960). Cellulolytic activity was expressed as μg glucose released (mg protein)$^{-1}$ min^{-1}. The protein content of the enzyme preparations was determined by Lowry's method using bovine serum albumin as a standard.

Materials. Substrates for cellulolytic activity were: carboxymethylcellulose (Sigma), xylan (larchwood; Sigma), D-(+)-cellobiose (Eastman Kodak, Rochester, N.Y., U.S.A.), ball-milled pulp (Solka-floc, BW200; Brown Co., Berlin, N.H., U.S.A.), absorbent cotton batting, filter paper (Whatman no. 1), microcrystalline cellulose (Avicel, pH 105; American Viscose, Marcus Hook, Pa., U.S.A.) and facial tissue paper.

Commercially available cellulase preparations from *Aspergillus niger* (Type II practical grade; Sigma) and *Trichoderma viride* (Type IV, crude lyophilized powder; Sigma) were used at the optimum pH (5·0) and temperature (37 °C) recommended for these preparations.

RESULTS AND DISCUSSION

Acetivibrio cellulolyticus contained a cellulolytic enzyme system capable of hydrolysing a range of cellulosic materials to reducing sugars (Table 1). The system in the culture supernatant acted on cotton batting and ball-milled pulp, both of which are composed of crystalline and amorphous celluloses, and on filter and tissue papers as well as cellobiose, carboxymethylcellulose and xylan. This indicated the production of endoglucanase, exoglucanase, cellobiase (β-glucosidase) and xylanase activities by this anaerobe. Comparison

between cellulolytic activities present in the culture supernatant and the cell-free extract indicated that a substantial proportion of these activities were cell-associated. For example, the cellobiase and filter paper activities in the cell-free extract were 7·5 and 2·5 times higher than in the culture supernatant.

The enzyme preparations were fairly stable at 2 °C and showed a pH optimum range between 5 and 6 and temperature optimum between 37 and 50 °C, depending on the substrate used. There was approximately a 5% loss in exoglucanase activity, a 15% loss in filter paper and xylanase activities and a 22% loss in endoglucanase activity after 4 weeks storage at 2 °C.

The results indicated that the cellulolytic activity of *A. cellulolyticus* was as good as or better than that of commercially available enzyme preparations from *Aspergillus niger* and *Trichoderma viride*. This was especially so for substances containing crystalline cellulose. Direct comparison of the cellulolytic activities of *A. cellulolyticus* with other anaerobes, such as *Clostridium thermocellum* (Ng *et al.*, 1977), or with aerobes and their mutants (Montenecourt & Eveleigh, 1977; Reese, 1977; Stutzenberger, 1971) is difficult because of differences in the use of substrate, test conditions, variation in protein content of culture fluids or enzyme preparations and variations in methods used for the estimation of sugars. However, the enzyme preparations from *A. cellulolyticus* were different from many described in the literature in that they attacked cotton fibres and possessed cellobiase and xylanase activities. Cell-free preparations from rumen anaerobes such as *Bacteroides succinogenes* and *Ruminococcus albus* do not attack cotton fibres (Halliwell & Bryant, 1963), while the cellulolytic enzyme system from *T. viride* is poor in cellobiase and xylanase activities (Ghose *et al.*, 1976; Herr, 1979).

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