NOTES

Carboxymethyl Cellulase and Cellobiase Production by *Clostridium acetobutylicum* in an Industrial Fermentation Medium

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The production of a carboxymethyl cellulase and a cellobiase by *Clostridium acetobutylicum* was demonstrated. In liquid medium the carboxymethyl cellulase was induced by molasses, and it was not repressed by glucose. Optimum carboxymethyl cellulase activity occurred at pH 4.6 and 37°C.

Before the discovery of synthetic processes for the production of acetone and butanol, these solvents were produced from corn starch or molasses by *Clostridum acetobutylicum* in an industrial fermentation system (8, 9, 11). As a result of the world energy shortage, there is renewed interest in the microbial process and the possibility of substituting less valuable materials for edible substrates. *C. acetobutylicum* is able to ferment pentoses (2, 4, 6), and its potential as an industrial strain for the utilization of agricultural wastes would be further enhanced if the bacterium was able to degrade cellulose. We describe the production of a carboxymethyl cellulase and a cellobiase by a *C. acetobutylicum* strain which is being used in the commercial production of acetone and butanol (11).

C. acetobutylicum strain P270 was supplied by National Chemical Products Ltd., Germiston, South Africa. The potato medium used for the maintenance of the *C. acetobutylicum* strain and the molasses fermentation medium (CFM) have been described previously (1). The clostridial basal medium (CBM) contained, in grams per liter of distilled water: glucose, 10; casein hydrolysate, 4; yeast extract, 4; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; *p*-aminobenzoic acid, 0.001; thiamine hydrochloride, 0.001; cysteine hydrochloride, 0.5; and biotin, 2 × 10^{-6}. The pH was adjusted to 6.7 with 0.1% (wt/vol) $NaHCO_3$. Incubation was at 34°C, and agar plate cultures were incubated in GasPak (BBL Microbiology Systems) jars. The anaerobic glove cabinet and techniques described by Moodie and Woods (5) were used for liquid cultures.

Cellulase, carboxymethyl cellulase, and cellobiase activities were determined in 1.0-ml supernatant samples by assaying reducing sugar equivalents released in 1 h at 37°C from 2.0 ml of sodium acetate buffer, pH 4.8, containing either 60-mg filture strips (Whatman no. 1) or 0.1% (wt/vol) carboxymethyl cellulose (BDH Chemicals Ltd., Poole, England) or 0.7% (wt/vol) cellobiose (Sigma Chemical Co.). Reducing sugar was estimated by the method of Nelson (7) as modified by Somogyi (10), using glucose as a standard. One unit of activity was defined as the quantity of enzyme required to catalyze the formation of 1 μmol of reducing sugar, expressed as glucose, per minute under the above conditions.

The production of a cellulase by the *C. acetobutylicum* strain was demonstrated by zones of clearing around isolated clones after 2 to 3 days of incubation on CBM-agar supplemented with 0.5 to 2.0% (wt/vol) acid-swollen cellulose powder (CF 11, Whatman), prepared by the method of Tansey (12). Although the degradation of acid-swollen cellulose occurred in agar plates and the bacterium digested cellulose dialysis membranes added to liquid cultures, no filter paper activity was detected in any of the liquid media tested. The clear zones on the CBM plates containing acid-swollen cellulose powder were not artifacts because a cellulase preparation, cellulysin (Calbiochem), added to wells in the plates produced similar zones. The diameter of the zones was proportional to the concentration of cellulase. A *Clostridium perfringens* strain did not produce zones of clearing on the medium. The *C. acetobutylicum* strain did not grow on CBM without glucose supplemented with either acid-swollen cellulose or Avicel as the sole carbon source.

However, both extracellular carboxymethyl cellulase and cellobiase activities were observed

in the liquid molasses fermentation medium (Fig. 1). Maximum cellobiase activity occurred before the pH breakpoint (24 h) at 20 h. The maximum levels of carboxymethyl cellulase activity were obtained after the pH breakpoint at 35 h. The cellobiase activity was unstable and decreased rapidly after 20 h, whereas the carboxymethyl cellulase activity was relatively stable over a 37-h period.

A novel discovery was that carboxymethyl cellulase activity required molasses for induction, and cultures containing decreasing amounts of molasses showed proportionately less carboxymethyl cellulase activity (Fig. 2). No induction of carboxymethyl cellulase or filter paper activity was detected in liquid CBM containing acid-swollen cellulose powder, Avicel, carboxymethyl cellulose, sucrose, glucose, cellobiose, or xylose. The substance in molasses which was responsible for the induction of carboxymethyl cellulase was a small molecule since dialyzed molasses failed to induce carboxymethyl cellulase activity. Molasses clarified by centrifugation at 10,000 × g for 30 min induced the production of carboxymethyl cellulase activity. The sediment after centrifugation did not induce carboxymethyl cellulase activity. The

molasses utilized was South African final molasses and contained approximately: dry solids, 81.5%; sucrose, 36.1%; reducing sugars, 14.5%; and sulfated ash, 15%.

The optimum pH for carboxymethyl cellulase activity in the culture supernatant was measured in different sodium acetate buffers and was shown to be pH 4.6. The optimum temperature for the enzyme activity was 37°C at pH 4.6.

The effect of glucose on carboxymethyl cellulase activity was determined by supplementing CFM medium with increasing concentrations of glucose (0.2 to 2.0% wt/vol). The addition of glucose did not affect the carboxymethyl cellulase activity, and the enzyme was not repressed by glucose.

The cellobiase was not specific for cellobiose and is a β-glucosidase because it released glucose from salicin. The cellobiase did not require induction by either molasses or cellobiose and was present in liquid CBM containing glucose. Localization studies involving washing and sonicating cells indicated that the cellobiase was extracellular, and no intracellular or cell-bound cellobiase activity was detected.

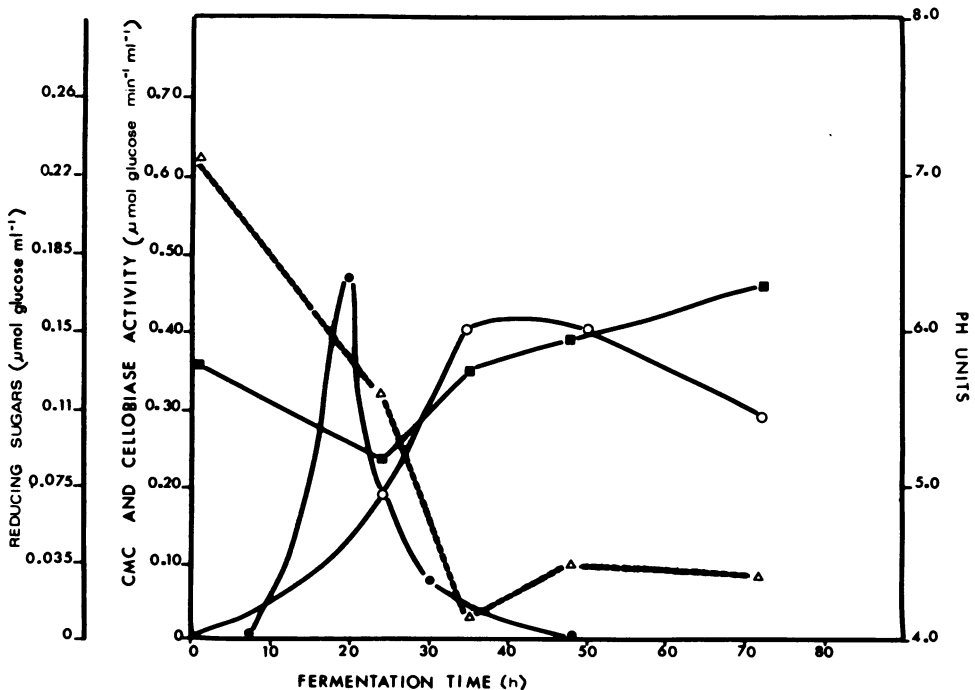The data from these experiments indicate that C. acetobutylicum possesses an inducible car-

FIG. 1. Production of carboxymethyl cellulase (CMC) and cellobiase enzymes by C. acetobutylicum in molasses medium (134 g of molasses per liter). Symbols: ○, carboxymethyl cellulase activity; ●, cellobiase activity; △, reducing sugars; ■, pH.
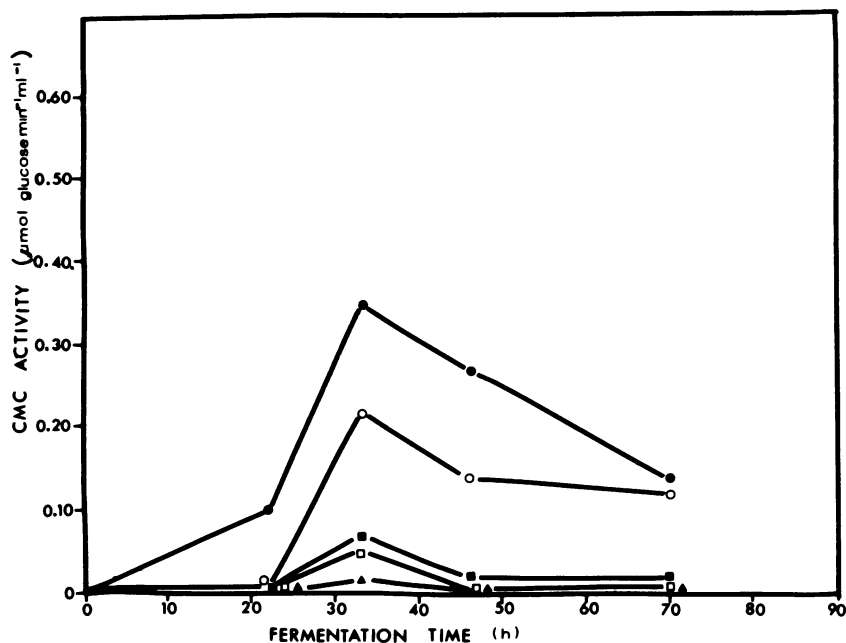
FIG. 2. *Induction of carboxymethyl cellulase (CMC) by molasses. Molasses medium (CFM) contained decreasing concentrations of molasses:* ●, *134;* ○, *67;* ■, *13.4;* □, *2.6; and* ▲, *0 g/liter. The media containing 13.4, 2.6, and 0 g of molasses per liter were each supplemented with 3.6, 10, and 10 g of sucrose per liter, respectively.*

boxymethyl cellulase and a cellobiase. In comparison with other cellulolytic bacteria and fungi, the levels of carboxymethyl cellulase and cellobiase activities obtained in *C. acetobutylicum* are low. The reason why acid-swollen cellulase is cleared on plates is presumably due to the carboxymethyl cellulose, as no activity against crystalline cellulose could be demonstrated. However, *C. acetobutylicum* does have the genes for carboxymethyl cellulase and cellobiase and the levels and activity could be improved by mutation and selection techniques (3). The cellulase required for crystalline cellulose degradation could be inserted by genetic manipulation (3).

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