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Pectinolytic activity of *Clostridium thermocellum*: Its use for anaerobic fermentation of sugar beet pulp

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Summary. *Clostridium thermocellum* is well known for its ability to convert cellulose into ethanol and to hydrolyse hemicellulose. The present work shows its ability to hydrolyse model pectins and to use them for growth. The main products on these substrates as well as on sugar beet pulps were as follows: acetate, ethanol and methanol. Galacturonase and lyase activities were measured in the fermentation broths. As shown by the accumulation of methanol in the medium, there is a pectin esterase activity but this one seems to be very low.

Introduction

Many studies have been made on cellulase activity of *Clostridium thermocellum* on model substrates (Gordon 1981; Zertuche and Zall 1982), but also on natural substrates such as sugar cane bagasses (Goel and Ramachandran 1983), corn stover (Wang et al. 1979) or exploded aspen wood (Saddler and Chan 1982). In Europe, sugar beet pulp is available in large amounts near sugar beet processing plants. The conversion of these residues into ethanol may be interesting in alcohol production.

Conversion of sugar beet pulp into ethanol and the hydrolysis of some of its polymers by this bacterium, were examined in the present study. The proportion of pectins in sugar beet pulps being large (about 20%), special attention was paid to the fate of this fraction.

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Material and methods

Strain and media. A clone of *Clostridium thermocellum* ATCC 27405 was cultivated on a modified CM_3-medium (NG et al. 1977) (5 g/l of yeast extract).

Flask culture. The medium was admixed with a buffer ($NaHCO_3$ 10 g/l) and then poured (250 ml) into 500 ml Flasks. This, and also the concentrated soluble carbon substrates (100 g/l) were flushed with nitrogen for 3 min, whereafter the flasks were closed with rubber stoppers. After sterilization (121° C, 20 min) carbon substrates (10% vol/vol) were added. The cultures were inoculated with 10 ml of a 24 h subculture (O.D. 2) and incubated at 60° C. Growth assays were made on flasks with commercial pectins (Unipectine, Paris) as carbon sources. Two types of pectins were used pectins "Rapid Set" (R.S.) (Methylation degree (M.D.) 71 to 75%) and pectins "Medium Rapid Set" (M.R.S.) (M.D. 65 to 70%).

Fermenter culture: Stainless steel fermenters (10 l) were used. Insoluble substrates were added to the medium (50 g/l of dry grinded sugar beet pulp). After sterilisation (121° C, 90 min), $NaHCO_3$ (3 g/l) was added to the medium which was flushed with nitrogen for 30 min to obtain anaerobiosis. The mixture was stirred at 200 rpm, at a temperature of 60° C and pH was maintained with 5 N ammonia at 7.0. Fermenter was inoculated with 250 ml of a 24 h subculture. After inoculation the fermenter was closed hermetically.

Samples were taken during the fermentation, an aliquot of each was frozen immediately and another part was filtered through paper (Whatman no. 1). For assays on sugar beet pulp a centrifugation (5 min, 1000 *g*) was performed before filtration to prevent obstruction of the filter. To determine growth, optical density (625 nm) was measured on the filtrate to follow the growth.

At the end of fermentation, thawed samples were centrifuged (4000 *g*, 30 min). The bottoms were dried (80° C, 24 h) and weighed to estimate the changes in the insoluble substrate. Pectins were extracted from the dried bottoms by the method of Barbier and Thibault (1982), four fractions of pectins were obtained: either extractible by water (WEP), oxalate (OEP), acids (HEP) or diluted alkali-(NaEP).

Galacturonic acid from the supernatant and each fraction of the total pectins were measured using the automated method of Thibault (1979).

Total reducing sugars in the supernatant were measured using Dinitrosalicylic Acid (DNS) (Miller 1959). Methanol,

ethanol and acetic acid produced by the fermentation were measured by Gas Liquid Chromatography analysis using a 3-metre glass column filled with SP 1220. Temperature of the injector was 140° C, that of the oven was 100° C at the moment of injection; a gradient of 2° C/min to 110° C was used. Helium flow rate was 16 ml/min. The sample was acidified with 10% of pure phosphoric acid. Butanol solution 8‰ (vol/vol) was added to the sample (1 vol/vol) as internal standard; 2 µl of this solution were injected into the chromatograph.

Measurement of enzyme activities. Pectin hydrolase activity of *C. thermocellum* culture supernatant was measured as follows: 1 ml of a polygalacturonic acid (1% in 0.1 M citrate buffer (pH 5.5)) was added to 1 ml of the enzymatic solution. The whole was incubated for 15 min at 60° C. Reducing sugars were then measured by DNS method (Miller 1959).

Pectin lyase activity was measured as described by Kapitonova et al. (1972) (Lyase activity corresponds to an increase of 0.1 O.D. unit at 237 nm in 60 min).

We did not succeed in measuring pectin esterase activity, but we measured the change in the proportion of acetylated pectins in each fraction of the extracted pectins (disesterification was made by incubating each extract admixed with 0.05 N NaOH vol/vol, at 4° C for 1 h. The acetate released by this treatment was measured by GLC. The value of control samples where water was used instead of NaOH was deduced from the value obtained.

Results

Three flask cultures of *C. thermocellum* were carried out with monogalacturonic acid; no growth was observed. However, on polygalacturonic acid this bacterium grows quite well (Fig. 1) and the drop of total galacturonic acid shows that *C. thermocellum* can use this substrate as a carbon source. Using this substrate the main product of fermentations of *C. thermocellum* was acetic acid and not ethanol as we observed with cellulose as substrate. In the former case molar ethanol/acetic acid ratio was about 0.1 at the same conditions, in the latter case, this ratio was 1.5.

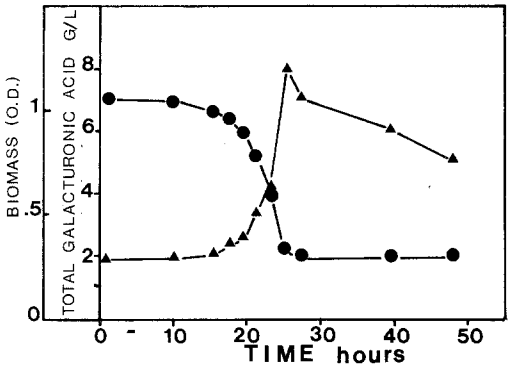

Fig. 1. Variation in biomass (▲) and polygalacturonic acid (●) during fermentation with this substrate, in flask (60° C)

C. thermocellum had a good growth on model pectins $\mu = 0.164\ h^{-1}$ on R.S. and $0.134\ h^{-1}$ on M.R.S. However, in the former case 60% of total galacturonic acid was assimilated in the later 80% was used. In both cases about 1.5 g/l of methanol were accumulated in the medium. As with the cultures with polygalacturonic acid the molar ethanol/acetic acid ratio was lower than with cellulose.

In the fermentation of sugar beet pulp we observed that a large part of the pulp is dissolved by sterilization and that up to 12% of the initial dry weight was to be found in solution, in the form of polygalacturonic acid. This part was quickly assimilated by *C. thermocellum* (20 h): 70% of the pectins and 70% of the insoluble substrate were used by this strain during 80 h of fermentation.

Enzymatic assays permit us to measure pectin hydrolase and pectin lyase activities of *C. thermocellum* (Fig. 2). *C. thermocellum* was able to dissolve

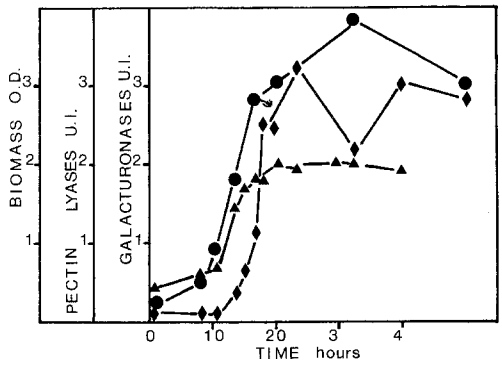

Fig. 2. Changes in pectin-lyases (●) and galacturonases activity (◆) and in biomass (▲) during fermentation of pectin "medium rapid set" in flasks

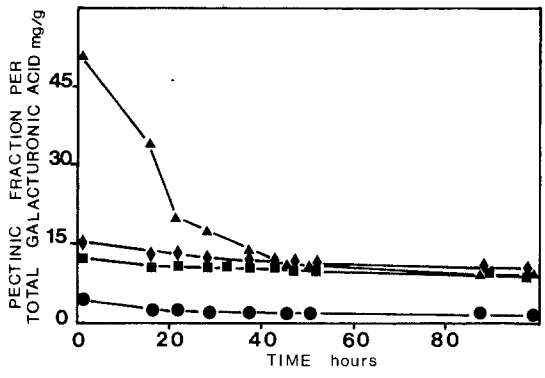

Fig. 3. Changes in pectinic fractions (per total galacturonic acid) in insoluble residues of sugar beet pulp during fermentation of this substrate in a 10 l reactor: pH 7.0; temperature 60° C; WEP (▲), OEP (●), HEP (■), NaEP (◆)

about 70% of a 5% solution of sugar beet pulp (Fig. 4). The maximum speed of decomposition obtained was 0.75 g/l/h. During decomposition of sugar beet pulp about 72% of galacturonic acid was degraded. Analysis of total galacturonic acid showed the rapid breakdown of these carbohydrates and the study of the change in the different pectin fractions in the insoluble substrate showed that only the WEP fraction was used by the bacterium: the other fractions are small in sugar beet pulp (3% of dry matter). The acetylated fraction of the pectins of sugar beet pulp was found into HEP and WEP fractions before sterilization. But the sterilization at a pH of about 5.0 dissolved 78% of the HEP fraction and acetylated residues could not be measured after sterilization in the solid phase. As in cultures with pectins the molar ethanol/acetic acid ratio was low (0.5). The amount of ethanol and acetic acid obtained from 50 g/l of sugar beet pulp was 3.2 g/l and 7.8 g/l. If only considering the hydrolyzed sugar beet pulp the yield of production of these two compounds were 10% and 25% respectively.

Discussion

C. thermocellum is not able to use monogalacturonic acid as carbon source, whereas it is able to use polygalacturonic acid and pectins as carbon source. It was already shown that *C. thermocellum* was more able to use cellobiose or cellulose than glucose because glucokinase is an inducible enzyme (Patni and Alexander 1971); Park and Ryu (1983) showed that this ability was bound to vitamins requirements of *C. thermocellum*. According to our results, this strain may hydrolyse cellulose, hemicellulose (xylanes) and pectins, owing to its enzyme equipment. This strain is thus able to hydrolyse simultaneously the major hydrocarbons of parietal polymers of sugar beet pulp.

Pectinolytic activity of several mesophilic anaerobic bacteria such as *Clostridium felsineum* (Avrova et al. 1981), *Clostridium multifermentans* (Sheiman et al. 1976), *Clostridium butyricum* (Obi 1981; Szymanski 1981) and a few thermophilic anaerobic bacteria such as *Clostridium thermosulfurogenes* (Schink and Zeikus 1983) and other strains of *Clostridium* and *Erwinia* (Schink and Zeikus 1980) have been studied. Our results showed that galacturonase and pectin lyase are both produced by *Clostridium thermocellum*. We could not measure in vitro pectin esterase activity, nevertheless release of methanol in the medium may be bound to this activity as shown by Schink and Zeikus (1980). The low activity of this enzyme might be responsible for the failure of our measurement assays. Despite the results of Lund and Brocklehurst (1978), who did not find any pigmentated *Clostridium* able to express the three types of pectinase activity at the same time, this strain seems to have this ability. Although the increase in galacturonase activity is associated with the growth phase and stops with it, the pectin lyase activity production begins later and goes on after the growth has stopped.

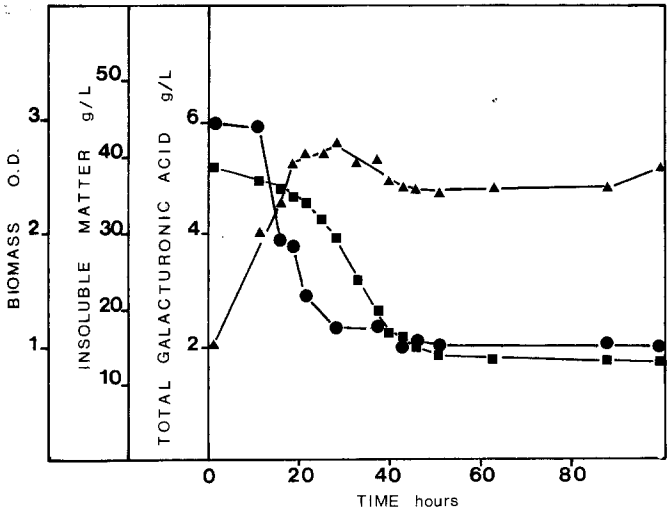

Fig. 4. Evolution of growth (▲) and substrates during culture in a 10 l reactor of *C. thermocellum*; pH 7.0; temperature 60° C; Total insoluble (■); total galacturonic acid (●)

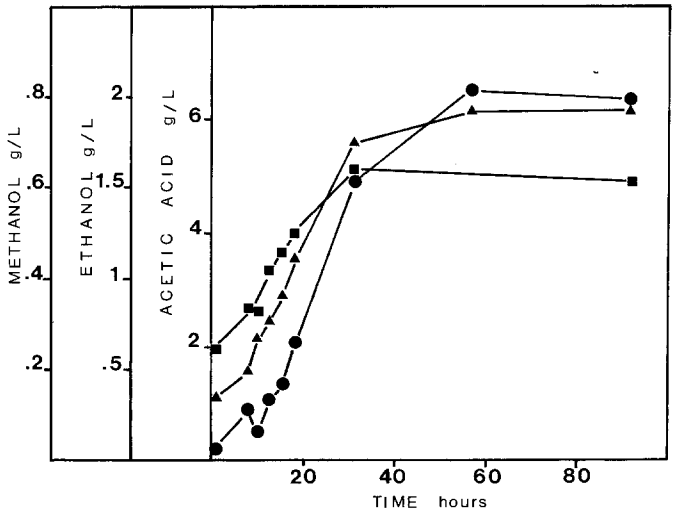

Fig. 5. Production of ethanol (●), acetic acid (▲) and methanol (■) during culture of *C. thermocellum* on sugar beet pulp. (Same assay as in Fig. 4)

The molar ethanol/acetic acid ratio is much lower on pectins and on sugar beet pulps than on cellulose. Initially, we suspected the acetilated pectins to be responsible for the difference observed, but the results obtained with polygalacturonic acid were the same as those obtained with pectins from this point of view. Because of the low concentration of acetylated residues bound to pectins before sterilization (a few ppm) it may be concluded that this acetylated fraction cannot be responsible of a lower molar ethanol/acetic acid ratio. This indicates that the metabolism of this strain is different with cellulose and with pectins.

Use of pectolytic enzymes to increase biodegradation of sugar beet pulp has already been studied by Popova et al. (1981). The pectinases were added to sugar beet pulp during extraction. The monomers released were then fermented by yeasts like sucrose and converted to ethanol. In our case sterilization costs, culture conditions of *C. thermocellum,* low yield of conversion of dry matter to ethanol, and low final concentration of ethanol are important drawbacks to industrial use of this bacterium.

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