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Thermophilic Methanogenesis from Pectin by a Mixed Defined Bacterial Culture

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Abstract. Thermophilic degradation of pectin was studied in batch cultures at 55°C by different associations of anaerobic bacteria, including *Clostridium thermocellum*, *Methanobacterium* sp., and *Methanosarcina* sp. *Clostridium thermocellum* alone produced large amounts of methanol along with some isopropanol and H_2. The inoculation of *Methanobacterium* sp. in the culture did not affect the metabolism of *C. thermocellum*; this demonstrates the absence of interspecies hydrogen transfer. In the presence of the methylotrophic *Methanosarcina* sp., methanol was reduced to methane without effect on pectin hydrolysis; a small amount of the H_2 produced was also used to reduce methanol.

Pectin, a major constituent of plant cell walls, is a polymer consisting mainly of poly α-(1,4)-galacturonic acid, partially esterified with methanol. Microbial pectin metabolism may generate significant amounts of methanol in nature, particularly during anaerobic digestion of fruit and vegetable wastes, because of the known activity of pectin methylesterase on methoxyl esters of galacturonic acid [4, 13, 15].

Under mesophilic conditions, pectinase activity has been shown in clostridia [8], especially in *Clostridium multifermentans* [21], *Clostridium felsineum* [1], *Clostridium butyricum* [16, 20], and in many other bacteria in the rumen [25, 28], such as *Lachnospira multiparus* [14]. The thermophilic pectinolytic activity of *Clostridium thermosaccharolyticum* [6], *Clostridium thermosulfurigenes* [18, 19] and *Clostridium thermocellum* [24] has been also investigated.

Studies of methanogenic fermentation of pectin have been reported in mesophilic conditions for a bacterial coculture [14, 17] or a mixed bacterial population [2]. Several results have been obtained with thermophilic mixed defined cultures on other organic substrates [11, 12, 22, 26].

In the present paper, we report results on thermophilic methanation of pectin, a large constituent (20%) of sugar beet pulp.

Materials and Methods

All chemicals were of reagent grade unless otherwise stated. Pectin from citrus fruits (Sigma, St. Louis, Missouri, USA) was used during this work. Gases were purchased from Airgaz (Marseille).

The organisms used were *Clostridium thermocellum* NCIB 10682, *Methanobacterium* sp., and *Methanosarcina* sp. [10] isolated in our laboratory.

The culture medium contained the following compounds (g/L): pectin (Sigma P-9135) 5; $(NH_4)_2SO_4$, 1.3; K_2HPO_4, 0.3; $MgCl_2 \cdot 6H_2O$, 1.0; $CaCl_2$, 0.15; L-cysteine-HCl, 0.5; yeast extract (Difco, Baltimore, Maryland, USA), 2.0; resazurin, 0.001 g; and 0.03 ml of 5% $FeSO_4$ solution. The medium was prepared anaerobically, as described previously [11]. The medium was dispensed in 20-ml aliquots into 60-ml serum bottles. N_2-CO_2 (80-20%) was the gas phase. After sterilization (110°C, 30 min) and just before inoculation, 0.2 ml of 2% $Na_2S \cdot 9H_2O$ and 0.9 ml of 10% $NaHCO_3$ were dispensed into each vial. The final pH was 7.0.

Stock cultures of bacteria and inoculations were made as previously described [22]. Average values of triplicate vessels are reported. Results are expressed per vial (20 ml medium).

The anaerobic culture techniques of Hungate [7] as modified by Macy et al. [9] were used throughout the course of this work. The incubation temperature was 55°C; H_2, CH_4, and volatile fatty acids were measured by gas chromatography [5].

Results

When grown on pectin, *Clostridium thermocellum* produced mainly methanol, with small amounts of isopropanol and H_2 (Fig. 1); no volatile fatty or or-

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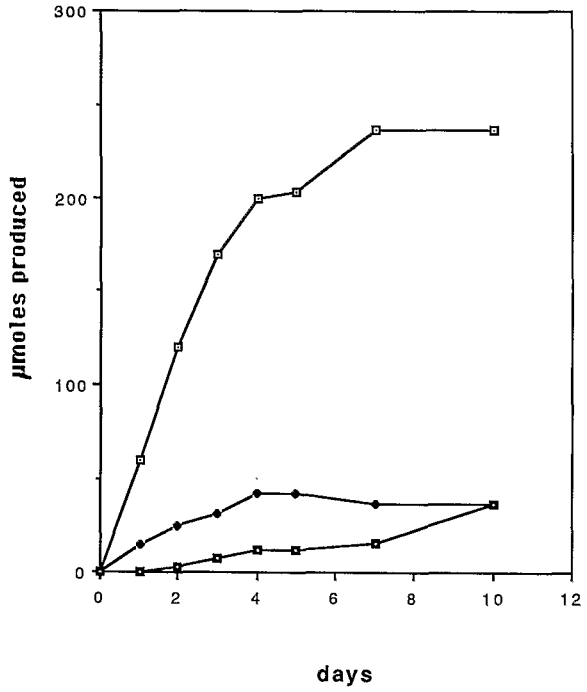

Fig. 1. Products of pectin hydrolysis by *Clostridium thermocellum* at 55°C. Results are expressed in µmol per 20 ml medium: methanol (⊡), isopropanol (●), H2 (■).

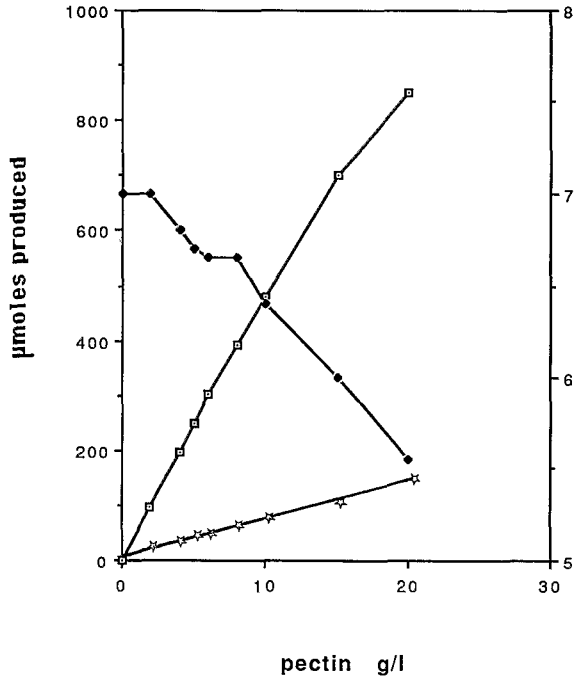

Fig. 2. Formation of methanol (⊡) and isopropanol (☆) and pH (●) evolution by *Clostridium thermocellum* growing at different initial concentrations of pectin. Data obtained after 10 days of culture at 55°C; µmoles produced are expressed per 20 ml medium.

ganic acids were detected. Increasing the pectin concentration in the medium led, after growth of *C. thermocellum*, to proportional amounts of methanol and isopropanol and lowered the pH to 5.5 when 20 g/L of pectin was introduced in vials (Fig. 2). Hydrogen was not proportional to the pectin concentration introduced (data not shown).

Clostridium thermocellum associated with *Methanobacterium* sp., an hydrogenotrophic methanogen, showed a very similar pattern, with production of CH_4 from the hydrogen generated by the pectinolytic bacterium (Fig. 3). The coculture of *C. thermocellum* with *Methanosarcina* sp., a methylotrophic methanogen, accumulated CH_4 from methanol, with partial utilization of H_2 evolved (Fig. 4; Table 1). Hydrogen was never detected by gas chromatography when *C. thermocellum* was cocultured with *Methanobacterium* sp. (Figs. 3–5), whereas a small amount of H_2 was measured when the pectinolytic bacterium was associated with *Methanosarcina* sp. (Fig. 4; Table 1). Furthermore, *Methanosarcina* sp. was unable to use all the methanol produced during hydrolysis of pectin (Figs. 4–5; Table 1).

In the triculture, the methane production was not significantly increased (Fig. 5; Table 1). Isopropanol always accumulated without subsequent consumption by the two methanogens.

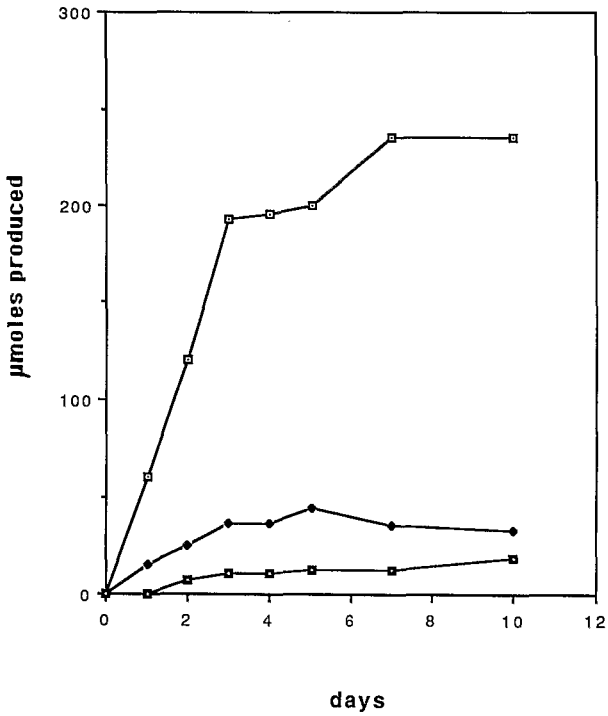

Fig. 3. Products of pectin hydrolysis by a coculture of *Clostridium thermocellum* and *Methanobacterium* sp. at 55°C. Results are expressed in µmol per 20 ml medium: methanol (⊡), isopropanol (●), CH_4 (■).

Table 1. Degradation products of pectin by various bacterial associations[a]

Culture	Methanol	Isopropanol	Hydrogen	Methane
C. thermocellum	237	36.7	39.8	0
C. thermocellum + Methanosarcina sp	43.6	36.7	5	188
C. thermocellum + Methanobacterium sp	235	33.2	0	13
C. thermocellum + Methanobacterium sp + Methanosarcina sp	45.4	30.1	0	195

[a] Results are expressed in μmoles per 20 ml of medium. Data obtained after 10 days of cultivation at 55°C with 5 g of pectin per liter.

Discussion

Spinnler et al. [24] were the first to report the pectinolytic activity of *Clostridium thermocellum*; however, in contrast to their strain, which produced moderate amounts of ethanol and acetic acid, strain NCIB 10682 accumulated only isopropanol. Isopropanol production from pectin hydrolysis has never been described for *C. thermocellum*, even in the last issue of Bergey's Manual of Systematic Bacteriology [3]. Nevertheless, a pectinolytic thermophilic *Clostridium* species (*C. thermosulfurigenes*) was found to produce isopropanol from pectin [18].

Our results showed that *C. thermocellum* possesses a pectin methylesterase that catalyzes the hydrolysis of methylesters linked with production of methanol and pectic (polygalacturonic) acids [23], as confirmed by the drop of pH in the culture medium. The production of methanol and isopropanol was proportional to the amount of pectin introduced. This proportional accumulation of methanol might be used for practical determination of pectin concentration in the medium described above, depending on methanol detected in *C. thermocellum* culture.

Isopropanol, a C-3 compound, could be produced from the C-6 component of the heteropolysaccharidic chain of pectin. The absence of volatile fatty acids and of ethanol, as well as the pH drop in the medium, indicated that *C. thermocellum* cannot ferment pectic acids. *C. thermocellum* NCIB 10682 probably has only deesterifying enzymes but not chain-splitting enzymes. Depending on the plant source of the pectin, more or less of the galacturonate residues may be acetylated at C-2 and C-3. Side chains of neutral sugar residues, mainly galactose, arabinose, and xylose, are covalently linked to C-2 or C-3 of the galacturonate residues [15]. *C. thermocellum* probably accumulated isopropanol by fermentation of these branched sugars, since it is already known that some *Clostridium* strains as *C. butylicum* are able to ferment sugars into isopropanol in a way similar to an acetone–butanol fermentation, except that the acetone is reduced to isopropanol [27].

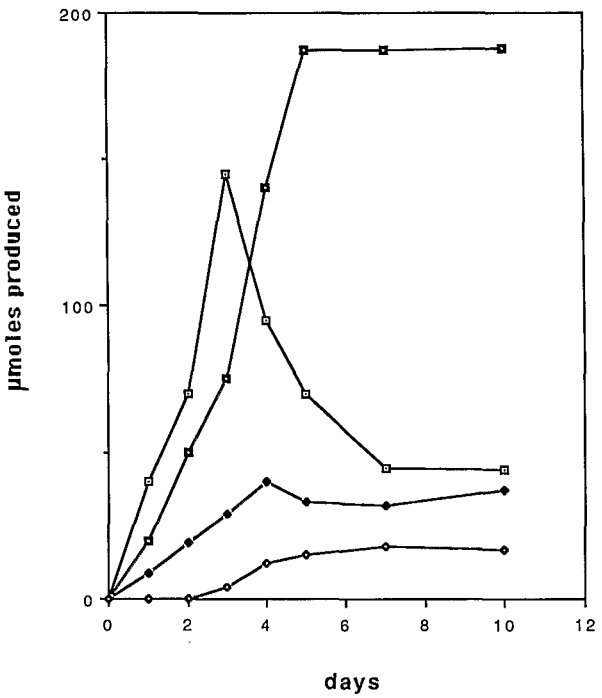

Fig. 4. Products of pectin hydrolysis by a coculture of *Clostridium thermocellum* and *Methanosarcina* sp. at 55°C. Results are expressed in μmol per 20 ml medium: methanol (⊡), isopropanol (●), CH_4 (■), H_2 (◆).

The association of *Methanobacterium* sp. with *C. thermocellum* did not modify the metabolism of the fermentative bacterium, indicative of the absence of interspecies hydrogen transfer between these two bacteria. Such a result has been obtained in mesophilic conditions, with *Eubacterium limosum* cocultured with *Lachnospira multiparus* grown on pectin [14]. The high affinity for H_2 of the thermophilic *Methanobacterium* sp. was responsible for the complete disappearance of hydrogen from the gas phase in the coculture with *C. thermocellum*.

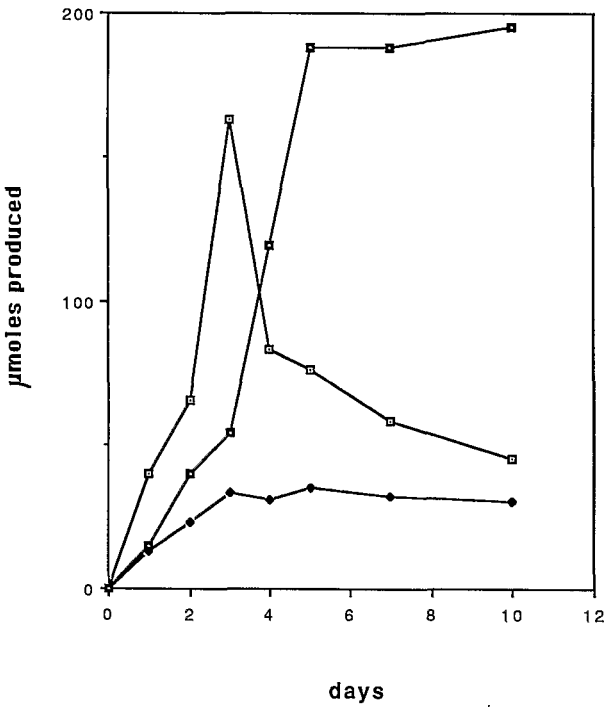

Fig. 5. Products of pectin hydrolysis by a coculture of *Clostridium thermocellum*, *Methanobacterium* sp., and *Methanosarcina* sp. at 55°C. Results are expressed in µmol per 20 ml medium: methanol (⊡), isopropanol (●), CH_4 (■).

The association of *C. thermocellum* with *Methanosarcina* sp. produced methane from pectin by the utilization of methanol by the methylotrophic methanogen. From the data of Table 1, a partial hydrogen consumption can be observed in the coculture, in comparison with the monoculture of *C. thermocellum*. *Methanosarcina* sp. was probably involved in the oxidation of hydrogen to reduce methanol as described for *Methanosarcina* TM1 [29], since our strain was unable to reduce CO_2 [10]. The use of methanol by the sarcina was a simple case of cross-feeding of a hydrolytic product of one organism to another.

In the triculture, H_2 was not detectable, because of the presence of *Methanobacterium* sp. Subsequent CH_4 production was not sufficient to modify significantly the amount of methane generated in the coculture clostridium–sarcina, because of limited quantities of hydrogen produced during the pectinolysis. Present experiments show that it is possible to accumulate methane from pectin under thermophilic conditions, by a defined mixed culture of three anaerobic bacteria. More than 95% of the methane produced can be obtained only by associating *C. thermocellum* with *Methanosarcina* sp.

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