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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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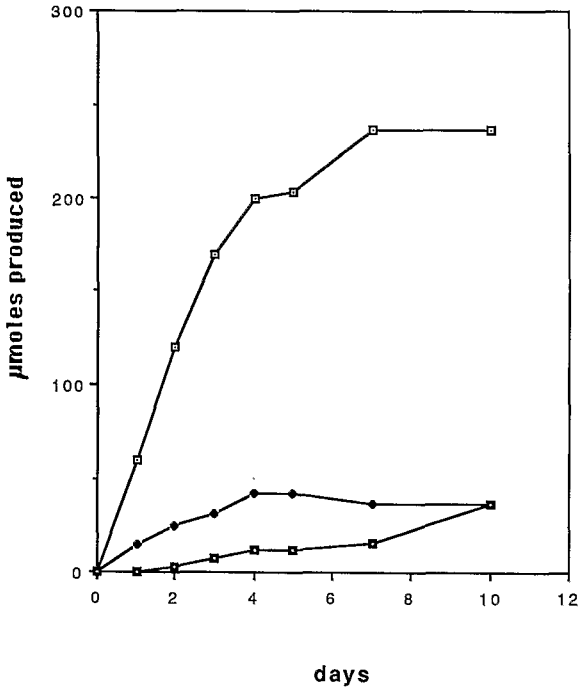

Fig. 1. Products of pectin hydrolysis by *Clostridium thermocellum* at 55°C. Results are expressed in µmol per 20 ml medium: methanol (⊡), isopropanol (●), H2 (■).

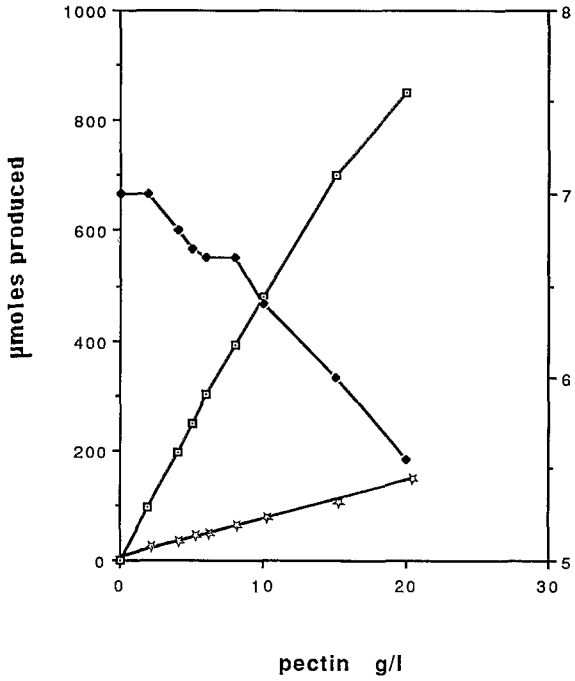

Fig. 2. Formation of methanol (⊡) and isopropanol (☆) and pH (●) evolution by *Clostridium thermocellum* growing at different initial concentrations of pectin. Data obtained after 10 days of culture at 55°C; µmoles produced are expressed per 20 ml medium.

ganic acids were detected. Increasing the pectin concentration in the medium led, after growth of *C.*

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. ther-*

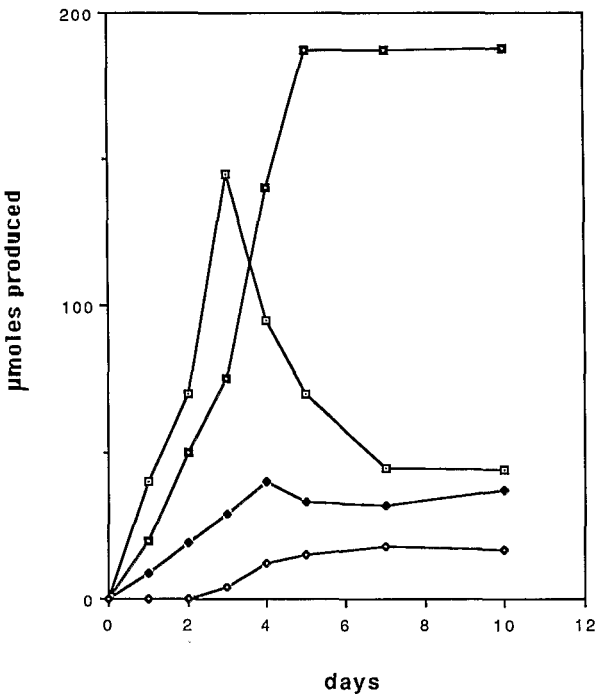

Fig. 4. Products of pectin hydrolysis by a coculture of *Clostri-*

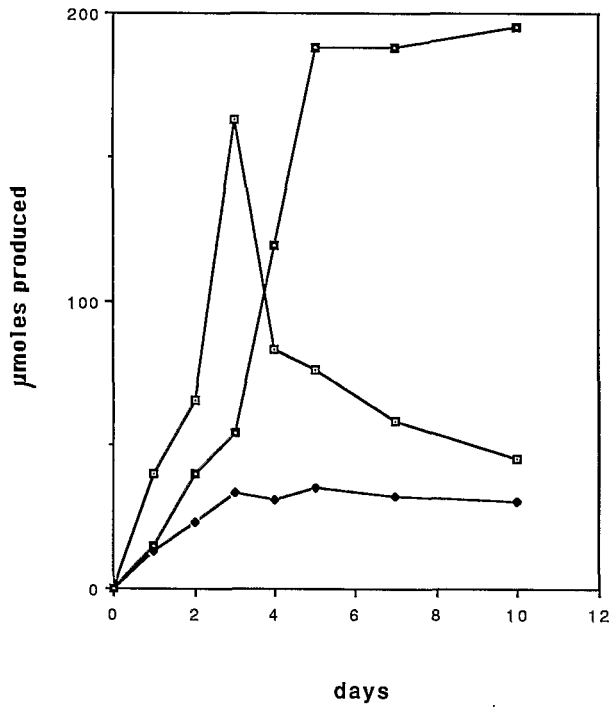

Fig. 5. Products of pectin hydrolysis by a coculture of *Clostridium thermocellum*, *Methanobacterium* sp., and *Methanosarcina* sp. at 55°C. Results are expressed in µmol per 20 ml medium: methanol (⊡), isopropanol (●), CH_4 (■).

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

Literature Cited

1. Avrova NP, Zubko IK, Alekseeva EG (1981) Fermentation products and pectinolytic enzyme activity of *Clostridium felsineum* strains having different rates of spore germination. Microbiologiya 3:318–325
2. Breure AM, Beerepoot M, Van Andel JG (1985) Acidogenic fermentation of pectin by a mixed population of bacteria in continuous culture. Biotechnol Lett 7:341–344
3. Cato EP, George WL, Finegold SM (1986) Genus *Clostridium*. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's Manual of systematic bacteriology, vol. 2. Baltimore: Williams & Wilkins, pp 1141–1200
4. Fogarty WM, Ward OP (1974) Pectinases and pectic polysaccharides. Progr Ind Microbiol 13:59–119
5. Garcia JL, Guyot JP, Ollivier B, Trad M, Paycheng C (1982) Ecologie microbienne de la digestion anaérobie: techniques de numération et d'isolement. Cah ORSTOM, sér Biol 45:3–15
6. Hollaus F, Sleytr U (1972) On the taxonomy and fine structure of some hyperthermophilic saccharolytic clostridia. Ark Mikrobiol 86:129–146
7. Hungate RE (1969) A roll-tube method for cultivation of strict anaerobes. In: Norris R, Ribbons DW (eds) Methods in microbiology, vol. 3B. New York: Academic Press, pp 117–132
8. Lund BM, Brocklehurst JF (1978) Pectic enzymes of pigmentated strains of *Clostridium*. J. Gen Microbiol 104:59–66
9. Macy JM, Snellen JE, Hungate RE (1972) Use of syringe methods for anaerobiosis. Am J Clin Nutr 25:1318–1323
10. Ollivier B, Lombardo A, Garcia JL (1984) Isolation and characterization of a new thermophilic *Methanosarcina* strain (strain MP). Ann Microbiol (Inst Pasteur) 135B:187–198
11. Ollivier B, Smiti N, Garcia JL (1985) Thermophilic methanogenesis from sugar beet pulp by a defined mixed bacterial culture. Biotechnol Lett 7:847–852
12. Ollivier B, Smiti N, Mah RA, Garcia JL (1986) Thermophilic methanogenesis from gelatin by a mixed defined bacterial culture. Appl Microbiol Biotechnol 24:79–83
13. Rexova-Benkova L, Marokovic O (1976) Pectic enzymes. Adv Carbohydr Chem 33:323–385
14. Rhode LM, Sharak Genthner BR, Bryant MP (1981) Syntrophic association by cocultures of methanol- and CO_2-H_2-utilizing species *Eubacterium limosum* and pectin-ferment-

20. Schink B, Ward JC, Zeikus JG (1981) Microbiology of wetwood: importance of protein degradation and *Clostridium* species in living trees. Appl Environ Microbiol 42:526–532
21. Sheiman MI, MacMillan JO, Miller L, Chase JR (1976) Coordinated action of pectinesterase and polygalacturonase lyase complex of *Clostridium multifermentans*. Eur J Biochem 64:565–572
22. Smiti N, Ollivier B, Garcia JL (1986) Thermophilic degradation of cellulose by a triculture of *Clostridium thermocellum*, *Methanobacterium* sp. and *Methanosarcina* MP. FEMS Microbiol Lett 35:93–97
23. Somogyi LP, Romani R (1964) A simplified technique for the determination of pectin methylesterase activity. Anal Biochem 7:498–501
24. Spinnler HE, Lavigne B, Blachere H (1986) Pectinolytic activity of *Clostridium thermocellum*: its use for anaerobic fermentation of sugar beet pulp. Appl Microbiol Biotechnol 23:434–437
25. Szymanski PT (1981) A note on the fermentation of pectin by pure strains and combined cultures of rumen bacteria. Acta Microbiol Pol 30:159–163
26. Weimer PJ, Zeikus JG (1977) Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl Environ Microbiol 33:289–297
27. Wilkinson JF, Rose AH (1963) Fermentation processes. In: Rainbow C, Rose AH (eds) Biochemistry of industrial microorganisms. London: Academic Press, pp 379–414
28. Wojciechowitz M, Tomerska H (1971) Pectic enzymes in some pectinolytic rumen bacteria. Acta Microbiol Pol Ser. A3:57–61
29. Zinder SH, Mah RA (1979) Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. Appl Environ Microbiol 38:996–1008