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Growth of *Clostridium thermobutyricum*: a Cellulolytic Thermophile

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Increased concentrations of yeast extract led to increased growth yields and faster growth rates of the newly isolated *Clostridium thermobutyricum*. This species produced butyrate as its main fermentation product from glucose as well as from yeast extract. In the presence of peptone or tyrptone and during growth on agar, up to 70% of the cells sporulated. Growth yields were 30 and 55 g per mole glucose in the presence of 0.05 and 2.0% yeast extract, respectively. The Arrhenius graph was biphasic, exhibiting an intermediary plateau around 38°C with a concomitant change in the Arrhenius energy. The optimum temperature was 55°C. An unusually sharp decline in the growth rate occurred above 59°C.

The formation of butyrate by thermophilic anaerobic cellulose degraders has been debated for a long time. Although validated in a 1926 publication (9), pure cultures of *C. thermocellum* were not obtained until more than 20 years later. McBee (5) showed clearly that butyrate was not a fermentation product of the cellulose degrader, but was a product of contaminating glycolytic organisms. Since then it has been assumed that butyrate is formed by *C. thermosaccharolyticum*, for a longtime the only thermophile known to form butyric acid in substantial concentrations, although not as the major product (1, 6, 11). Enebo (3) described another thermophilic cellulose degrader, *C. thermocellulaseum*, which was believed to produce large amounts of butyrate. However, it was later found that butyric acid was produced by a contaminant which Enebo isolated. Unfortunately, both cultures were lost. During attempts to reisolate Enebo's cellulose degrader, a similar butyrate producer was isolated and it was named *C. thermobutyricum* (12).

Fermentation of glucose by this species, in the presence of yeast extract, can be represented by the following equation: Glucose→0.85 butyrate+1.8 CO_2+1.9 H_2+0.2 lactate+0.1 acetate. Ratios of butyrate to glucose above 0.9 have been observed, but butanol was not formed in detectable (>0.3 mM) concentrations in these experiments. The growth requirements of *C. thermobutyricum*, its response to varying temperatures and growth yields during growth on glucose and yeast extract are described here.

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Effect of Temperature on Growth

Growth of *C. thermobutyricum* occurred between 26 and 61.5℃ with an optimum temperature (T_{opt}) of around 55℃. A decrease in growth rate above the optimum temperature occurred in an unusually narrow temperature interval, i.e., from approximately 90% of the shortest doubling time to no growth at all within a 2℃ range (Fig. 1). Furthermore, between 35 and 40℃, a transition in the Arrhenius Energy occurred. To ensure that this was not a mixed culture, the organism was grown at both 30 and 60℃ and subsequently single cell colonies were isolated from each culture. The t_d at several temperatures for each culture grown from these cells was then determined. Each culture had the same doubling time (e.g., 0.90 and 0.88 h at 56℃; 1.57 h at 39℃; and 1.80 and 1.75 h at 37℃). The temperature curve displayed with these cultures was repeated and it was found that each one exhibited the biphasic curve. This indicated that a significant change in the rate limi-

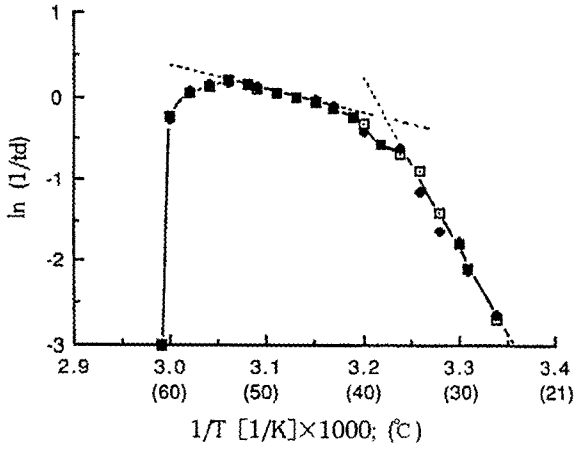

Fig. 1. Arrhenius graph (natural log of growth rate versus the reciprocal of temperature in °K).
Cells were grown in mineral medium containing 0.3% yeast extract and 0.5% glucose at a starting pH of 7.0. At each temperature, the culture was allowed to adapt for at least 5 generations before growth rate was determined in a subsequent subculture by following the increases in optical density at 600 nm. Values represent a combination of two independent experiments (—◙—; —◆—) each with two parallels.

ting step occurs in the temperature range between 35 and 40℃. Such biphasic curves are known for several extreme thermophiles such as *Thermoanaerobacter ethanolicus* (13), *C. thermohydrosulfuricum* (11), *Methanobacterium thermoautotrophicum*, and *Bacillus stearothermophilus* (4, 8, 10). All of these organisms exhibit a growth span of 40℃ or more and thus are called temperature tolerant extreme thermophiles (10). *C. thermobutyricum* exhibits a temperature span of approximately 35℃. However, with a minimum temperature of 26℃, it grows at a much lower temperature than other thermophilic anaerobes such as *C. thermocellum*, *C. thermosaccharolyticum*, *C. thermoaceticum* and *C. thermoautotrophicum*. Therefore, according to Wiegel and Ljungdahl (10), this organism is regarded a temperature tolerant thermophile.

Yeast Extract Requirement

Yeast extract was required for the substantial growth (Fig. 2a~c) of this organism. When glucose concentrations were held at 0.5% (w/v), and yeast extract concentrations were increased up to 5% (w/v), the cell densities increased. In the absence of glucose, yeast extract served as the sole carbon and energy source. The optical density of the culture increased linearly with an increase in the yeast extract concentration between 0.05 and 2%. With increased yeast extract concentrations of 0 to 0.3, the doubling time decreased markedly, regardless of whether

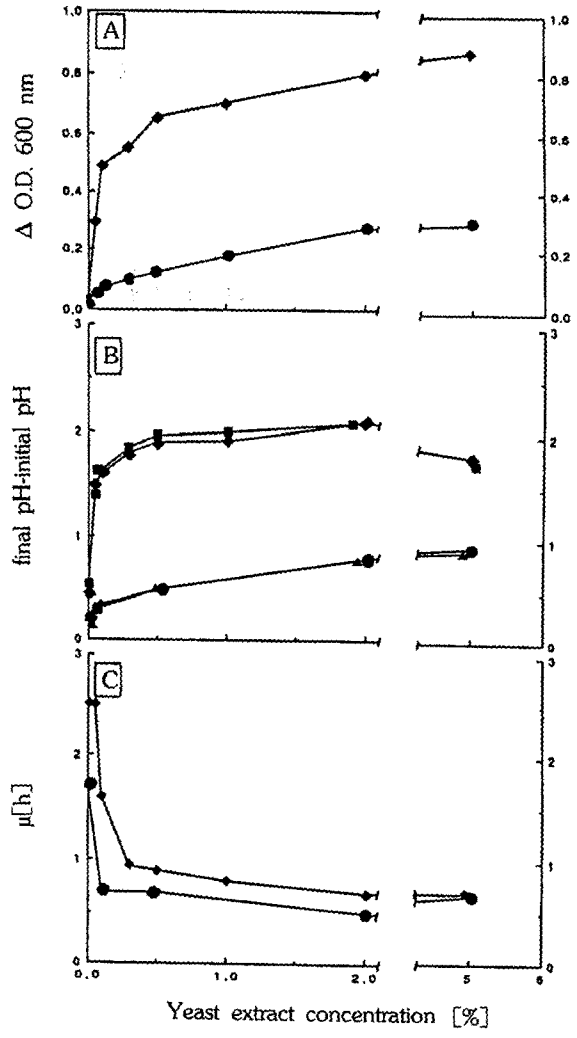

Fig. 2. Growth dependence on yeast extract in the presence and absence of glucose.
Growth temperature was 57℃; pH 7.0. The graphs show yeast extract versus a) optical density at 600 nm; b) maximal doubling time; c) delta pH (final pH-initial pH). The values represent the means of two independent experiments with two parallels each: ([—■—; —◆—] with addition of 0.5%, w/v, glucose; [—●—; —▲—] in the absence of glucose. the differences between the two experiments—688 in panel a and c—were within the symbol sizes and thus were not shown).

C. thermobutyricum was grown in the presence or absence of glucose (Fig. 2b). Although the maximum optical density reached in the absence of glucose was much smaller than in its presence, the organism grew faster on the yeast extract alone than in the presence of 0.5% glucose. This effect became more pronounced using 0.3 % yeast extract, but the same effect was not observed when using 2% yeast extract. In the presence of 0.05%

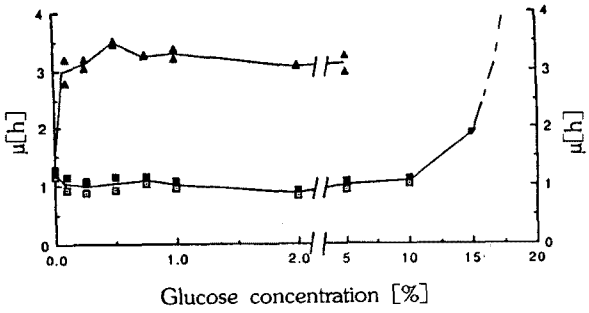

Fig. 3. Effect of glucose concentration on the doubling time in the presence of 0.05% (−▲−; −△−) and 2.0% (−◙−; −■−) yeast extract.
The values are means of two independent experiments, each with two parallels. The doubling time in the absence of glucose was determined in the range of 0.0~0.4 $OD_{600\ nm}$, whereas the others were obtained in the range of 0~0.6/7.

yeast extract, growth occurred in the presence of glucose concentrations at a rate as high as 5% (w/v), while in the presence of 2.0% yeast extract, glucose concentrations as high as 10% were tolerated with no increase in doubling time. Growth was inhibited above 25% glucose (Fig. 3).

Sporulation

In liquid cultures using 0.5 glucose, yeast extract (0~2 %) had no effect on sporulation frequency, which was below 1% under these conditions. This low sporulation frequency contrasted with that which had been observed in cellulolytic enrichment cultures and the frequency which had been observed during the early stages of isolation (12). In contrast to *C. thermoaceticum* and *C. thermohydrosulfuricum*, a slow decrease in the temperature from 20℃ above to 20℃ below optimal temperature did not induce a high sporulation frequency as expected from the biphasic Arrhenius plot (11). However, 30% of the cells sporulated after 3 days of growth on agar, regardless of the presence or absence of 0.3% yeast extract and/or 0.3% glucose or cellobiose. During the determination of the substrate spectrum (12), the addition of peptone and tryptone led to 70 and 40% sporulation, respectively. Neither peptone nor tryptone supported growth, nor did either one serve as a substitute for yeast extract. Furthermore, the addition of 20% of the culture supernatant from the purified cellulose degrader (taken from the original enrichment culture) to a growing culture led to a 5~10 fold increased in sporulation frequency. The compound(s) in the culture supernatant or in peptone and tryptone that led to higher sporulation rates were not determined.

Substrate Yield ($Y_{glucose}$)

The relationship of biomass produced per unit of glu-

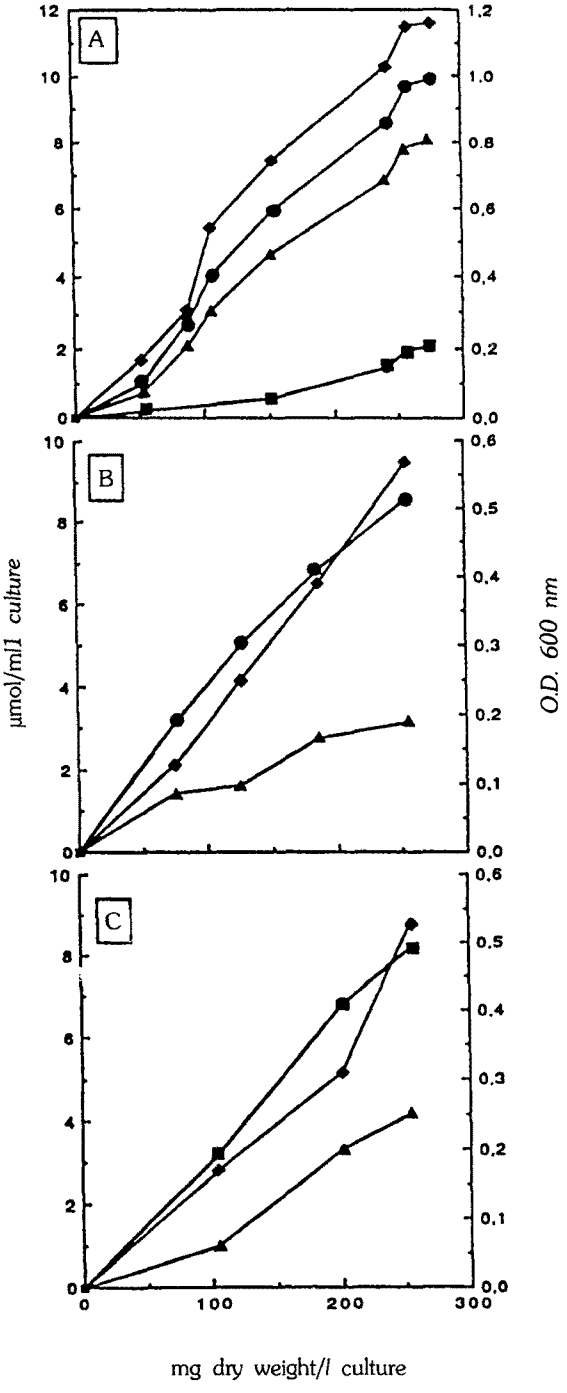

Fig. 4. Relationship between substrate utilization, product formation, absorbance and dry weight determination.
Cells were grown in mineral medium with an initial pH of 7.0 and which contained (a) 2.0% yeast extract plus 0.3% glucose; (b) 0.05% yeast extract plus 0.3% glucose and (c) 2.0% yeast extract with no glucose added. (−◆−) $OD_{600\ nm}$; (−●−) glucose utilized, (−▲−) butyrate formed; (−■−) lactate formed.

cose utilized with butyrate and lactate formed at 0.1% and 2% yeast extract plus/minus 0.3% glucose were determined. In several of these experiments, the acetate concentration was below 1 μmol/ml, and thus too low for this analysis to obtain exact values. Under all three conditions, close correlations between substrate used, product formed, optical density, and biomass produced were observed (Fig. 4). Growth yields were calculated to be 55 and 30 g per mole of glucose in the presence of 2.0% and 0.05% yeast extract. Assuming that *C. thermobutryicum* uses the usual clostridial pathway for butyrate formation, the utilization of glucose should lead to the formation of 3 ATP per mole butyrate (and 1 ATP per mole lactate or 2 ATP per mole acetate). Under this assumption and the fermentation balances obtained, the Y_{ATP} of 10.5 g from the research of Baushop and Elsden (2) appeared to be in agreement with the obtained value of 30 g dry weight per mole glucose utilized in the presence of 0.05% yeast extract indicating the yield of about 3 mole of ATP per mole of glucose utilized. Furthermore, it was postulated that the high value of $Y_{glucose}$ obtained in the presence of 2% yeast extract was due to the utilization of compounds in the yeast extract such as carbon and energy sources, as well as due to a relative higher Y_{ATP} compared to the one found in a less complex medium (7). During growth on 2% yeast extract (no glucose added) a Y_{ATP} of 12.5 g dry weight per mole ATP was obtained by using the data (Fig. 4C) that showed 16 mmole of butyric acid (corresponding to 48 mmole ATP) and 32 mmole of lactic acid (corresponding to 32 mmole ATP) were produced for 1 g of dry weight cells per liter. The corresponding Y_{ATP} value obtained in the same way for the fermentation of 0.5% glucose in the presence of 2% yeast extract was around 21 g dry weight cells per mole ATP.

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