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Monitoring growth and acetic acid secretion by a thermotolerant *Bacillus* using conduction microcalorimetry

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SUMMARY

A method is described to determine power of heat-time curves by conduction microcalorimetry in order to monitor the viability and ability of a thermotolerant *Bacillus* strain to secrete acetic acid both during exponential growth and during stationary-phase. In this system secreted acetic acid is neutralized by an insoluble source of lime (dolime) which results in a poor correlation between optical density and culture dry weight. As an alternative, cells and residual dolime were rapidly resuspended in isothermal fresh medium with glucose in a conduction microcalorimeter. Heat evolution was rapid over a period of 200–800 s. Steady state heat evolution rate decreased as a function of culture time and did not correlate with: 1) specific growth rate; 2) viable cell number; 3) glucose consumption rate; or 4) acetic acid secretion rate. Glucose consumption and acetic acid secretion during the stationary growth phase were correlated with specific heat evolution rate. These initial results indicate that this technique may be useful for further development as an on-line flow or stopped-flow method to monitor the physiology of bacilli in response to nutrient depletion or growth inhibition.

INTRODUCTION

Monitoring of *Bacillus* fermentation processes for the production of organic acids, amino acids, enzymes and expression of heterologous peptides is currently accomplished by measurement of oxygen uptake rate, CO_2 evolution rate, pH, and depletion of a growth-limiting substrate. Determination of cell density and peak cell viability by plate counting methods is too slow for process monitoring and optimization. Often, bacilli rapidly lose viability or initiate pre-sporulation processes at the end of exponential growth due to nutrient limitations [9,19,32]. The inability to rapidly monitor cellular activity for optimization of nutrient feeding is further complicated when insoluble media components make optical or filtration methods of cell density determination not feasible [23]. Development of high cell density batch or fed-batch aerobic *Bacillus* processes is often slowed due to the lack of a sensitive method to monitor changing cellular physiology. New methods are needed in order to better monitor the physiology of endospore-forming bacilli in response to nutrient depletion or accumulation of growth-inhibiting products such as organic acids in order to optimize high cell density batch or extended fed-batch processes [19, 32]. New methods useful for monitoring *Bacillus* fermentation processes that can be operated at 50–65 °C are needed. Exploitation of thermotolerant or thermophilic *Bacillus* for large-scale processes can result in significant energy savings for reactor temperature control, reduction in cooling capacity, as well as enhanced reaction rate [1].

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Calorimetric monitoring of microbial physiology

Heat evolution methods using thermograms or power of heat-time curves are useful methods for monitoring cell physiology in some fermentation processes for control of substrate feeding due to the considerable heat evolution from microbial metabolism of carbohydrates [7]. This method can be particularly useful for optimization of cell mass yield if the rate of heat evolution can be correlated with the growth rate, cell number or substrate consumption rates [7, 25,35]. Heat evolution methods have been reported for the determination of the number of metabolically active cells using mass and energy balances [35]. Correlation of the rate of heat evolution has also been observed for: organic substrate consumption; cell mass production; O_2 consumption; NH_3 consumption; and CO_2 evolution [8]. These measurements are useful where accurate measurement of heat evolution from the entire reactor is available. Accurate measurement of small changes in heat evolution for small volume aerobic fermentations is, however, often difficult [35].

Examples of calorimetric monitoring and control of fermentation processes using adiabatic calorimeters, isothermal calorimetry or temperature scanning calorimetry have been reported [2,7,8,21,22,25,35] using either continuous or stopped-flow methods [35,38]. Correlation of heat dissipation in order to monitor the exhaustion of nutrients, salts, or oxygen [35] or to control glucose feeding [25] has

been reported for *E. coli, Klebsiella, Streptococci*, and yeast. Although potentially scaleable from small volume to large scale reactors, sampling and sample conditioning are significant problems in routine application of these methods on-line. Off-line calorimetric methods have been used to monitor small scale fermentations [35], or the entire fermentation is conducted within a calorimeter [3,4,29,30,35].

Conduction microcalorimetry has been found to be a sensitive method for determination of the metabolism of monosaccharides and aromatic compounds by *E. coli* [27], to monitor cell number and cell viability [14], and to analyze the level of unreacted substrate in fermentations [28]. Recently, Boe and Lovrien, [5,6] have developed a conduction microcalorimeter method to determine the steady state heat output of *E. coli* to correlate with cell number as a rapid method to monitor cell mass during exponential growth.

In spite of the demonstrated usefulness of conduction calorimetric methods for determination of the viability of Gram-negative microorganisms, no reports of the application of calorimetry for monitoring of *Bacillus* processes have been published. Here we report an initial evaluation of conduction microcalorimetry for determining whether a correlation between heat evolution and cellular activity exists that could be used off-line to monitor growth or acetic acid secretion by a thermotolerant, spore-forming *Bacillus* species [34] in the presence of insoluble dolime. Intermittent feeding of dolime neutralizes accumulating acetic acid forming useful high magnesium acetate salts [38].

MATERIALS AND METHODS

Bacillus *strains and growth media*

A glutamate auxotroph mutant (C-27) of the thermotolerant *Bacillus* species C4 (ATCC 55182) [34] was used. Glutamate auxotrophy was selected in order to inhibit metabolism of accumulated acetic acid. *Bacillus* C-27 was grown in a chloride-free minimal salts medium (Table 1) in a 3L-aerated fermentor (Applikon, Inc.) operated at 53 °C in a circulating waterbath [34]. Low pH was necessary for dolime dissolution and was achieved by maintaining the pH at 5.0–5.2 by a pH controller (Applikon, Inc.) by intermittent addition of a 4% slurry of dolime (Miracle Lime, Pressure hydrated type 'S', Western Lime and Cement). The maximum specific growth rate of *Bacillus* C-27 on minimal glucose medium (Table 1) at 53 °C was 0.88 h^{-1}. Rapid exponential growth was complete 3 h after inoculation of 1.5 L of medium (Table 1) with 50 ml of a 6-h inoculum culture grown on the same medium incubated on a 55 °C shaker (Labline) at 350 rpm. Fermentor pH, dissolved oxygen tension, agitator speed (900–1000 rpm) and temperatures were continuously monitored on a strip chart recorder (model 4001, Chessel, Newtown, PA) and the data transferred to a personal computer for storage [34].

Conduction microcalorimetry

A conduction microcalorimeter [26,27] with dual 7 ml gold reaction vessels located between Seebeck devices was used in a 55 °C incubator and modified using a 180 degree reciprocating motor (15 cycles min^{-1}) to continuously mix the contents of the calorimeter cells by rotation. Heat flow from the Seebeck devices on the reference cell was subtracted from the test cell.

Calorimeter response was converted to specific heat evolution by dividing by the cell dry weight as previously determined in growth medium without added dolime [34]. The calorimeter was periodically calibrated with a small resistance heating coil inserted into the sample reaction chamber at 55 °C, 53 °C and 23 °C. From this data, the Figure of Merit was consistently determined to be 5.0 ± 0.10 μw/μv.

2 ml of culture broth containing 0.2–1.5 mg cell dry weight and insoluble dolime were removed from the fermentor and immediately resuspended in carbohydrate-free media (Table 2) pre-incubated at 55 °C, centrifuged, loaded into one

TABLE 1

Growth media for *Bacillus* C-27 (in 1 L of distilled water)

	grams
Glucose	5–30
Glutamate	1.5
K_2HPO_4	0.96
KH_2PO_4	32
Yeast Extract (Difco)	6.0
$(NH_2)SO_4$	8.0
$Ca(NO_3)_2$	1.65
$MgSO_4 \cdot 7H_2O$	2.46
10% (wt/vol) soln, of equal quantities of burning lard oil, herring oil, and corn oil	2 ml
Chloride-free trace salts soln.	1 ml
Chloride-free trace salts solution (g L^{-1}):	
$FeSO_4 \cdot 7H_2O$	2.0
$CuSO_4 \cdot 3H_2O$	0.04
H_3BO_3	0.03
$MnSO_4 \cdot H_2O$	0.15
$ZnSO_4 \cdot 7H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.047
$CoSO_4 \cdot 7H_2O$	0.047

TABLE 2

Media for suspension of *Bacillus* C-27 for microcalorimetry (in 1 L of distilled water)

	grams
Cell reservoir (total volume 1 ml)	
Glutamate	1.5
K_2HPO_4	0.96
KH_2PO_4	32
Substrate reservoir (total volume 2 ml)	
Glucose	30
Yeast extract (Difco)	6.0

compartment of the calorimeter cell and allowed to equilibrate for up to 15 min prior to mixing with the other compartment of the cell which contained fresh growth media (containing yeast extract) and 1 ml of a 30 g L^{-1} glucose solution. Particular care was required to rapidly resuspend and equilibrate the cells in the calorimeter in media pre-incubated to 55 °C at the same pH as the fermentation in order to minimize loss of cell viability and lysis. *Bacillus* C-27 cells held in an oxygen-deficient environment for a prolonged period of time prior to analysis rapidly lost viability. Abrupt changes in medium pH or temperature during centrifugation and resuspension also resulted in cell death. Control of temperature, oxygen supply and pH were required in order to minimize variability in determination of the specific heat evolution from one fermentation to another.

Transient heat evolution above that of the reference cell was recorded on a personal computer. Data were adjusted by subtracting any initial background voltage and smoothed by fitting to an 11th order polynomial which was used to calculate the slope and integral of the curve. Slope and integral were converted to watts (w), watt/sec (w/s) and calories (cal) by multiplication by conversion factors. The end of each test was determined as the time where the slope decreased to 0.1 mv s^{-1}. The total glucose consumed during the calorimeter test was less than 0.5% of the glucose added to the sample cell.

Determination of Bacillus *viability*

Viable cell concentration was determined by plate counting [24,36] on minimal salts medium containing glucose, yeast extract and 30 g L^{-1} Difco agar. Plates were incubated for 24 h at 55 °C. The correlation between cell dry weight and absorbance of 1.00 at 600 nm and the dry weight of carefully washed cells grown in medium not containing dolime was determined to be 261 mg L^{-1} cell dry weight. Glucose consumption and acetate accumulation were determined by HPLC as previously described [34].

RESULTS

Correlation between viable cell number and culture dry weight

Viable cell number in the presence of dolime could be determined only by plate counting. It was not able to be approximated from total culture dry weight due to the poor correlation with the total dry weight of cells + unreacted dolime (Fig. 1). Cell dry weight in the presence of dolime could not be predicted accurately using the optical density measured for cultures grown without dolime. The light scattering contributed by suspended dolime particles could be eliminated by adjusting the pH of each sample to >pH 12 with 1 N HCl, however, high pH adjustment resulted in cell death and lysis.

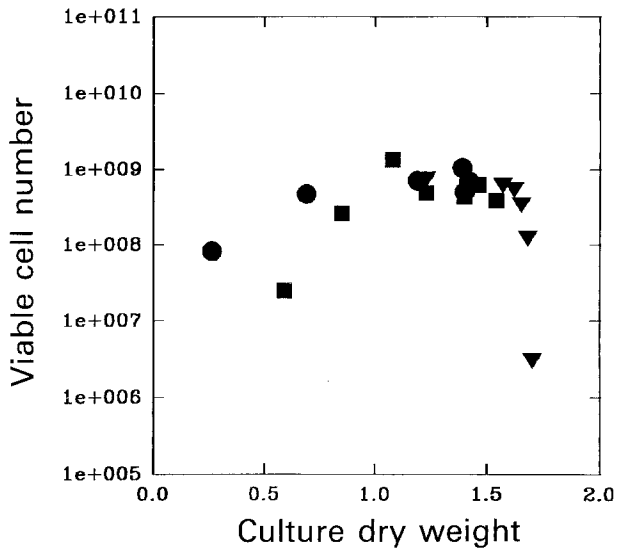

Fig. 1. Correlation between todal dry weight (cells + insoluble dolime) and viable cell number during growth of *Bacillus* C-27 in 1.5-L fermentations with pH control at pH 5.2 by intermittent addition of dolime. Fermentation media contained either (●) 2 g L^{-1} or 6 g L^{-1} (■, ▼) of yeast extract.

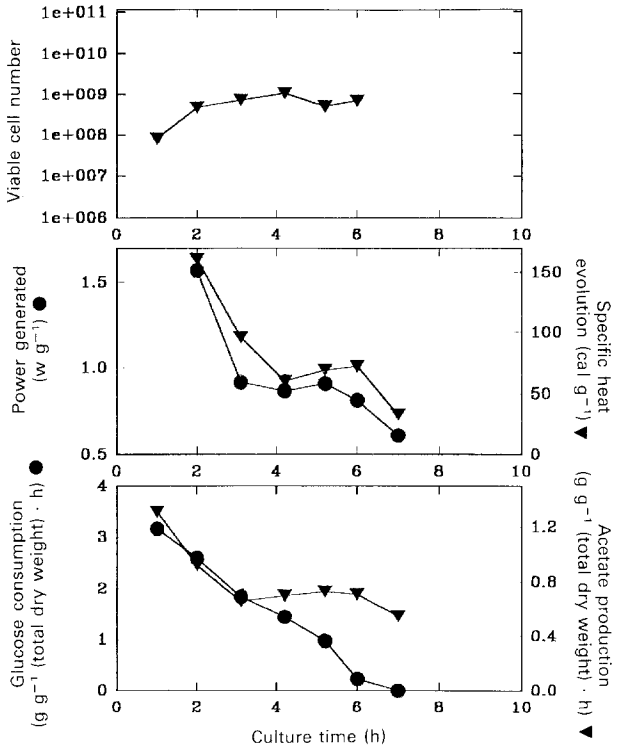

Fig. 2. Cell viability, specific heat evolution, glucose consumption and acetate production of *Bacillus* C-27 during batch culture at 53°, pH 5.2, with medium containing 2 g L^{-1} yeast extract.

Acetic acid secretion and steady state heat evolution secretion by Bacillus *C-27*

Acetic acid secretion by *Bacillus* C-27 was both growth and non-growth associated (Fig. 2) [34]. Specific acetic acid secretion rate decreased at the end of exponential growth but remained constant during stationary-phase in spite of the continued decrease in glucose consumption rate.

Samples were withdrawn from the reactor each hour and

power of heat-time curves generated. Total heat determined for each sample was determined from the final height of the steady state portion of the heat generation curve using the criterion of an end point of a slope of less than 0.1 mv sec^{-1}. Total heat generated per cell was calculated by integration. The slope of the initial rate of heat generation (Fig. 3) was found not to correlate with the specific growth rate, cell viability, glucose consumption rate or acetic acid secretion. The viability of cells following incubation in the calorimeter was not determined since calorimetric measurements were extended in order to determine the steady state response (heat evolution rate of <0.1 mv s^{-1}). In some instances, no steady state was reached (Fig. 3A) indicating that cells remained viable during the calorimetric measurement. The total heat generated by utilization of the quantity of glucose consumed in the calorimeter during the period of incubation was in the range predicted [33].

Rapid heat evolution over a period of 400–800 s was observed using this microcalorimeter method with *Bacillus* C-27 (Fig. 3). No initial transient spike of heat evolution followed by an extended steady-state level was observed [6]. Heat evolution was observed to rapidly increase to a steady state, often with a steadily decreasing slope (Fig. 3). Cells in the early stage of rapid exponential growth (1.0 h following inoculation, Fig. 3A) often did not reach a steady state but continued to evolve heat at a rate of change greater than 0.1 mv s^{-1} well beyond 800 s. This may be due to continued cell division in the calorimeter cell.

Both glucose consumption and specific acetic acid secretion were independent of heat evolution rate during exponential growth (Figs 4A, 5A). Maximum specific growth rate varied from 0.68 to 0.88 h^{-1} and did not correlate with steady state heat evolution rate. The ability of *Bacillus* C-27 cells to remain viable, continue glucose consumption and secrete acetic acid was positively correlated with steady state heat evolution during stationary-phase (Figs 4B, 5B). The specific rate of heat evolution during stationary-phase per gram of glucose consumed was approximately 56.5 cal g^{-1} CDW. The acetic acid secretion rate could be reasonably

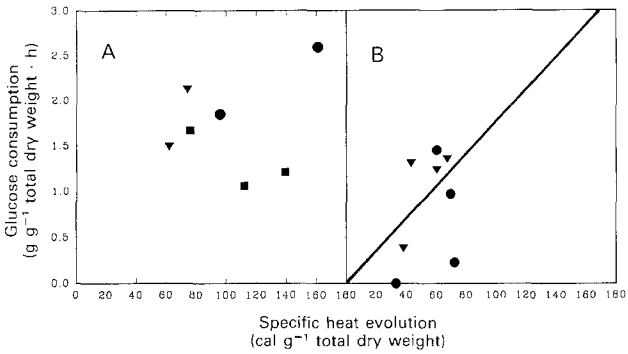

Fig. 4. Correlation of glucose consumption with heat evolution rate for *Bacillus* C-27 during: A) exponential growth; and B) during stationary-phase. Cells resuspended in fresh medium after being removed from a 1.5-L batch culture containing either 2 g L^{-1} (●) or 6 g L^{-1} (■, ▼) of yeast extract.

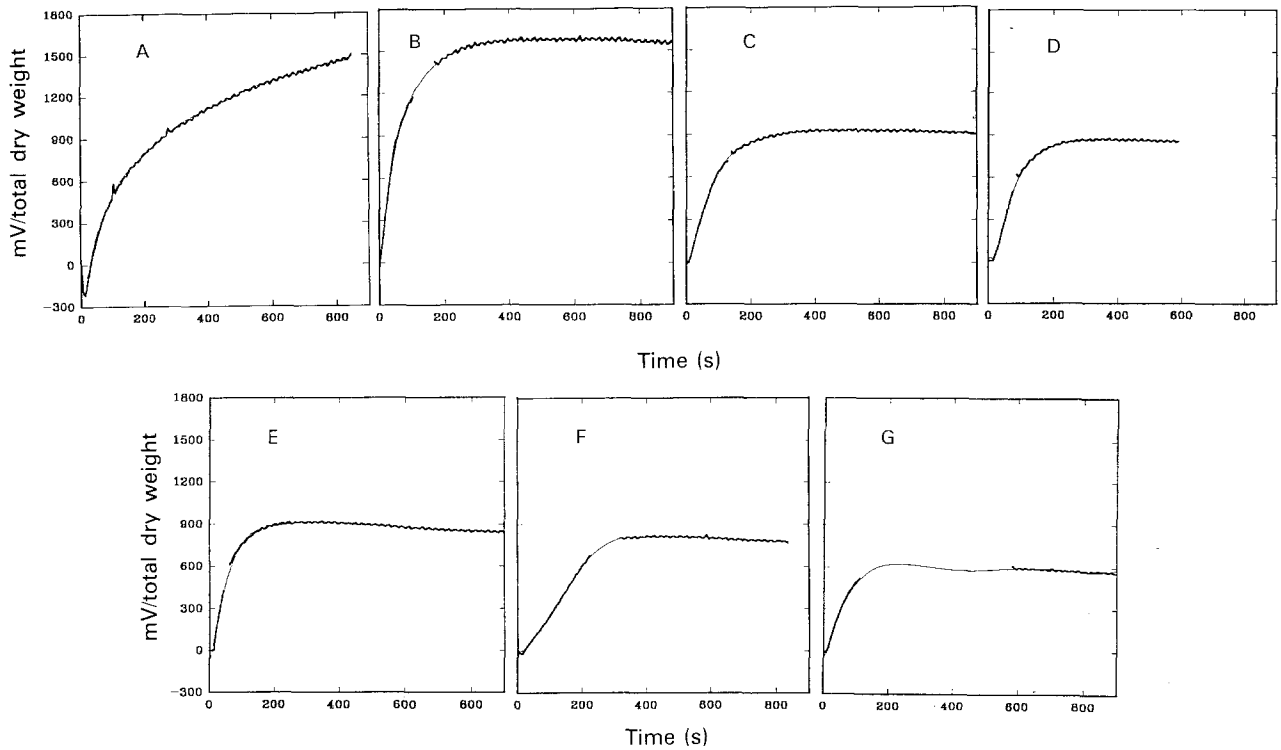

Fig. 3. Calorimetric response of *Bacillus* C-27 cells removed from a batch culture at 53 °C, pH 5.2. Time points correspond to those in Fig. 2. Panel A, 1.0 h; B, 2.0 h; C, 3.1 h; D, 4.2 h; E, 5.2 h; F, 6.0 h; G, 7.2 h. Each determination contains 1000 points shown in bold overlaid with an 11th order polynomial.

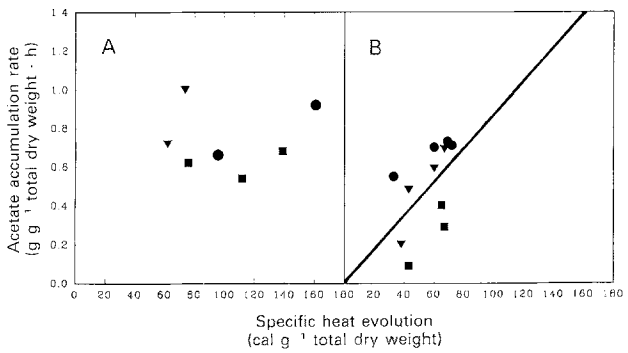

Fig. 5. Correlation between specific heat evolution rate and acetic acid secretion rate during: A) exponential growth; and B) stationary-phase. Symbols correspond to those in Fig. 4.

predicted from the specific heat evolution rate by using a specific rate of steady state heat evolution rate of 91.4 cal g^{-1} CDW (Fig. 5B). The data used to obtain these correlations (Figs 4 and 5) were forced through the origin since it was assumed that stationary-phase cells not consuming glucose would not be evolving heat.

DISCUSSION

Inhibition of growth and non-growing associated acetic acid secretion

Growth inhibition is a major difficulty in optimization of high cell density cultures for production of organic acids such as the production of acetic acid for vinegar or acetate salts for environmental applications [39]. Acetic acid production is both growth and non-growth associated [13]. It is, therefore, important to be able to monitor bacterial growth as well as the accumulation of acetate beyond cessation of rapid exponential growth in order to determine the onset of acetic acid inhibition of further acid secretion. Determination of acetic acid inhibition is usually accomplished by analysis of acid secretion kinetics. No method to directly monitor cellular activity following the cessation of growth in order to determine the potential for maintaining high specific acid secretion rate has been reported. Such a monitoring method would be very useful for optimization of cell growth to high cell density to optimize reactor volumetric acid secretion rate and to monitor inhibition of non-growth associated acetic acid secretion.

Approaches to optimize the conversion of carbohydrates to acetic acid using aerobic *Acetobacter* strains have used specialized aeration devices (Frings Acetator, Frings America, Naperville, Ill), fixed film and shallow plug-flow bioreactors [37], or cells immobilized in carrageenan gel beads in a fluidized bed [31]. Metabolic models based upon substrate, product, cell mass, and nucleic acid determinations are also being investigated [20] in order to describe and optimize growth and non-growth associated acid accumulation. Alternative genetic approaches to minimize the effect of acetic acid inhibition of cell growth are by manipulation of aldehyde dehydrogenase [11] or citrate synthase enzyme levels [12,34].

Acetic acid accumulation is rapid in aerobic bacteria because of the coupling of the generation of cellular energy with either the conversion of ethanol to acetic acid (*Acetobacter aceti, A. polyoxogenes*) [11,13] or as a metabolic side product in bacilli during rapid growth. The major portion of the energy needed by *Bacillus cereus* and *Bacillus subtilis* during vegetative growth is supplied by oxidation of glucose to acetic and pyruvic acids [15,17,18]. Further metabolism of the accumulated acetate by the activity of citrate synthase, aconitase, succinic dehydrogenase, fumarase and malic dehydrogenase is repressed during vegetative growth and derepressed following exhaustion of glucose prior to sporulation [17,18]. The tricarboxylic acid cycle in aerobic bacilli is also repressed by glutamate in the presence of glucose [10,16,36]. The accumulation of acetate and its further metabolism is a key indicator of *Bacillus* energy metabolism and transition from vegetative growth to sporulation.

Calorimetric monitoring of acetic acid secretion in Bacillus *C-27*

Although the data are limited, a method has been developed to obtain preliminary evidence that microcalorimetry may be useful to monitor cellular activity and continued acetic acid secretion by *Bacillus* strains during stationary-phase. These calorimetry results indicate that heat evolution by *Bacillus* C-27 is very rapid, that steady-state heat evolution data can be obtained, and that specific heat evolution decreases with decreasing growth rate. Steady-state heat evolution did not correlate linearly with maximum specific growth rate. *Bacillus* C-27 steady-state heat evolution was proportional to the decrease in cell growth rate, however, in contrast to *E. coli* [6], no strong linear correlation was observed between *Bacillus* viable cell number or specific growth rate and heat production. Also different from *E. coli*, steady-state heat evolution appeared to be independent of glucose consumption during exponential growth. This result is not surprising due to the requirement of yeast extract by this *Bacillus* for growth [34] and therefore a lack of a defined medium with a single growth-limiting nutrient. Steady-state heat evolution was independent of the yeast extract concentrations used in this study (2 or 6 g L^{-1}). A reasonable positive correlation between glucose consumption and acetic acid secretion rate was observed following the cessation of rapid exponential growth.

In general, the calorimetric response observed for this *Bacillus* (Fig. 3) is strikingly different than reported by other investigators for Gram-negative bacteria, streptococci or yeast [6,7,35]. In particular, the *E. coli* heat evolution-time curves of Shoda [35] show multiple changes in slope depending upon whether the cultures were glucose, nitrogen or oxygen-limited. This was not observed for *Bacillus* C-27. This difference may be the result of: 1) the strong dependence of this *Bacillus* on oxygen to maintain activity and viability; or 2) the transition in the physiology of *Bacillus* C-27 from vegetative growth to sporulation. *Bacillus* C-27 cells were

not observed to form spores during incubation in the calorimeter, however it cannot be ruled out that pre-sporulation processes may have begun during this incubation. Completion of sporulation during calorimetry, however, would have resulted in a peak of heat evolution followed by a rapid decline to a very low rate. This was not observed in any of the determinations. The sensitivity of heat evolution to decreasing growth rate may also indicate that calorimetry may be a very useful sensitive method to monitor changes in the physiology of bacilli when nutrient exhaustion occurs long before a switch from vegetative growth to sporulation can be microscopically observed.

Approaches for improving conduction microcalorimetry for monitoring bacilli

Fresh isothermal medium was used in this study to determine the maximum potential of the cells to continue to metabolize glucose to acetate during stationary-phase. An alternative approach would be to incubate the cells with the same media as present in the reactor at the time of sampling in order to mimic on-line calorimetric measurements. Under these latter conditions, the effect of accumulating acetate on cell growth and acetic acid secretion rate may be more easily observed.

The sensitivity to oxygen deprivation of this rapidly growing *Bacillus* strain during thermal equilibration may limit off-line applications of this method. On-line application of this approach using a stopped-flow incubation could reduce the difficulty of sample preparation and minimize loss of viable cells. A more rapid off-line or on-line stopped-flow calorimetric approach may be feasible by reducing the volume of the calorimeter cell compartment and the size of the Seebeck devices from the original design [6,26,27]. The 7-ml cell volume used in this study required long thermal equilibration times (5–15 min). Thermal equilibration time could be reduced significantly by using a very small volume cell (<1 ml), smaller Seebeck devices, and pre-equilibration of nutrients. These modifications would increase the sensitivity of this method for monitoring bacilli so that a more accurate correlation could be made between the time-course of specific heat evolution following cessation of rapid growth and 1) glucose depletion, 2) specific acetate secretion rate, or 3) maximum vegetative cell metabolic activity prior to initiation of sporulation or cell death. This type of sensing device could be particularly useful for optimization of intermittently fed *Bacillus* processes so that the substrate feeding rate could be altered in order to sustain rapid *Bacillus* vegetative growth to high cell density and cell viability.

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