Microcalorimetric Study of the Biological Effects of Zn^{2+} on *Bacillus thuringiensis* Growth

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A microcalorimetric technique was used to investigate the influence of Zn^{2+} on the growth metabolism of *Bacillus thuringiensis*. LKB-2277 Bioactivity Monitor was employed to obtain the power-time curves, from which the maximum peak-heat output power (P_{max}) in the log phase, the growth rate constants (k), the inhibitory ratios (I), the generational time (t_G) and the total heat effect (Q_{total}) in 23 h for the growth metabolism of *Bacillus thuringiensis* at 28 ℃ can be evaluated. The results indicate that the concentration of Zn^{2+} affects its growth obviously. Low concentration (0—50 μg/mL) of Zn^{2+} promotes the growth of *Bacillus thuringiensis* while high concentration (50—500 μg/mL) of Zn^{2+} inhibits its growth. When the concentration reached up to 600 μg/mL, it can not grow at all.

Keywords *Bacillus thuringiensis*, Zn^{2+}, growth metabolism, microcalorimetry

Introduction

Calorimetry has been used in monitoring cellular metabolism by means of heat measurements, especially in studying metabolism in cells and whole organism.[1,2] Microcalorimetry is also used in measuring the effects of various substances and culture conditions on metabolism. Miles and Beezer demonstrated that microcalorimetric studies of bacterial growth revealed temporal details which can not be observed by other techniques.[3] Microcalorimetry can also be used to study the metabolism of mitochondria and the effects of toxicants on mitochondrial metabolism.[4-8] Thermogenic curves contain a lot of kinetic and thermodynamic information. By analyses of the thermogenic curves, the effect of toxic agents on microbes[9,10] and mitochondrial metabolism have been studied,[11] the considerable kinetic data were obtained.

Zinc plays an important role in living system. Zn-enzyme exists in six enzymes, Oxidoreductases, Transferases, Hydrolases, Lyases, Isomerases and Ligases. Up to the date, about 300 kinds of Zn-protein have been found from the different species, which carry out more than 20 kinds of functions. Accordingly, Zinc is almost involved in any aspects of metabolism and absence of Zinc can break the balance of metabolism and cause a lot of diseases.[12]

Bacillus thuringiensis is one of the major microbial insecticides. It plays a major role in the shift of microbial insecticides. It is a Gram positive soil bacterium, and it belongs to Bacillus according to taxology of bacteria. *Bacillus thuringiensis* is widely applied to control crop

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pests, fruit and horticulture pests as well as medical and storage pests, and get remarkable ecological benefit.[13] Therefore, the study of the *Bacillus thuringiensis* metabolic process is important for the research on mode of action of this valuable species.

Experimental

Materials

Cells and reagents

Bacillus thuringensis YBT-1520 was provided by Key Laboratory of Agricultural Microbiology of Chinese Agriculture Ministry, Huazhong Agricultural University, Wuhan, China.

$ZnCl_2$ was of analytical grade and it was supplied by Zhonglian Reagent Factory, Beijing, China.

Cultural medium

B. thuringensis was grown on a LB medium which was made by taking tryptone (10 g), beef extract (5 g) and NaCl (10 g) in 1000 mL of water solution, pH = 7.0—7.2, and sterilized in high pressure steam at 120 ℃ for 30 min.

Calorimeter

The LKB-2277 Bioactivity Monitor, which is a type of heat conduction microcalorimeter, was used to determine the metabolism of cells. It was designed to monitor continuously a wide variety of processes and complex systems over the temperature range from 20 ℃ to 80 ℃. Each measuring cylinder normally contains a sample and a reference in separate measuring cups (twin system). The heat output from the sample flows through the thermoelectric detector to the large heat sink (in close contact with the water bath). In response, the detector produces a voltage, which is proportional to the power output from the sample. In order to minimize the systematic error and disturbance effect, a differential or twin detector system was used. This system is very sensitive, which the detection limit is 0.15 μW and the baseline stability (over a period of 24 h) is ±0.2 μW. There are three operating modes for the LKB-2277 Bioactivity Monitor: ampoule mode, flow-through mode and flow-mixed mode.

In the monitoring system, two precision resistors for electrical calibration were built into each measuring cylinder and one for each detector. When a known electronic current was passed through the appropriate resistor, the detector can be calibrated easily. Other methods for calibration were suitable internally calibrated radioactive sources and chemical reactions. Using one of these techniques, a calorimetric constant can be evaluated. The time constant (τ) of this instrument is about 120 s. The performance of this instrument and the details of its construction have been previously described.[14]

Preparation of sample

In this experiment, the solution of $ZnCl_2$ was prepared in sterilized distilled water and prepared freshly every time. At the beginning of the experiment, *B. thuringensis* was inoculated in the prepared beef extract medium, initially containing 1×10^6 cells/mL, and the cells used were suspended in the beef extract culture medium, then the fresh $ZnCl_2$ solution of 20, 50, 100, 200, 300, 400, 500 or 600 μg/mL was added into the cell suspension respectively.

Experimental procedure

Put the solution, LB medium (5 mL) containing *B. thuringiensis*, into stainless steel ampoule. And hook the lifer on the ampoules, then lower them slowly to the heat equilibration position and let the ampoule stays at this position for 30 min for pre-heating. After that, lower it slowly to the measurement position. Run the chart recorder, and the monitor will record the power-time curves of bacterial growth.

When the pen of the chart recorder is returned to the stable baseline, *B. thuringensis* growth will be ended. If necessary, further calibration was done after a stable baseline has been obtained.

Results

Thermogenic curves

The growth thermogenic curve of *B. thuringensis* in LB medium at 28 ℃ was shown in Fig. 1, and the thermogenic curves of *B. thuringensis* growth effected by $ZnCl_2$ were shown in Fig. 2.

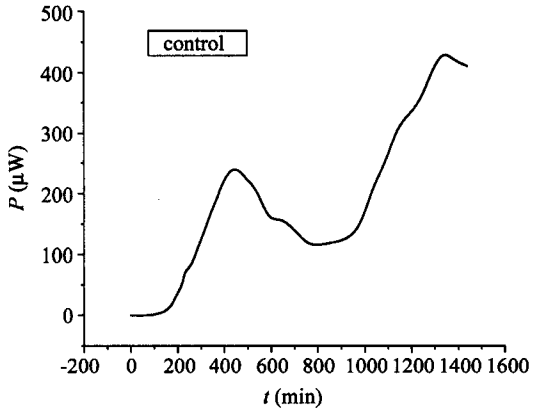
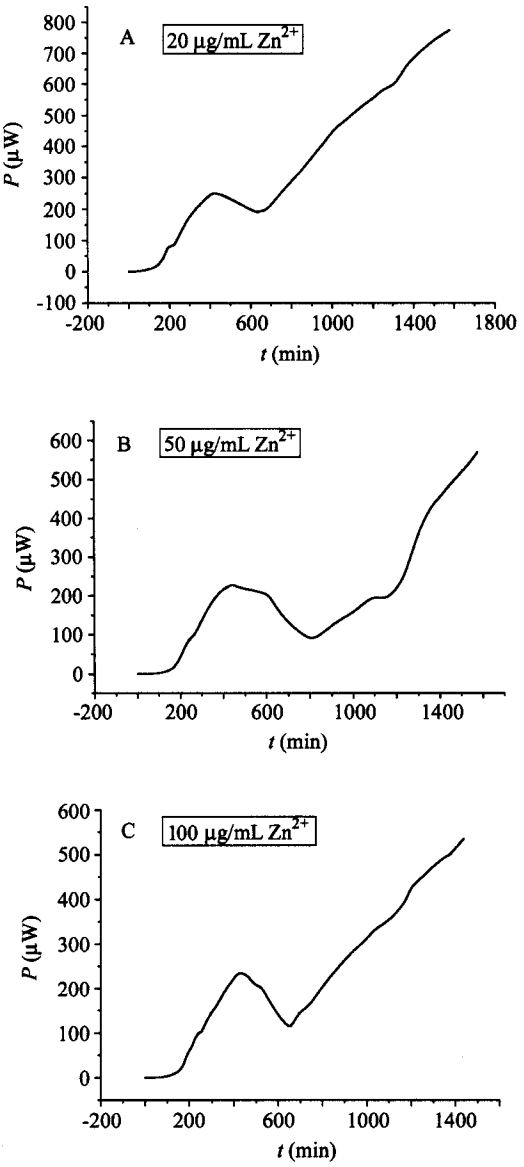


Fig. 1 Growth thermogenic curve of *B. thurigiensis* at 28 ℃.



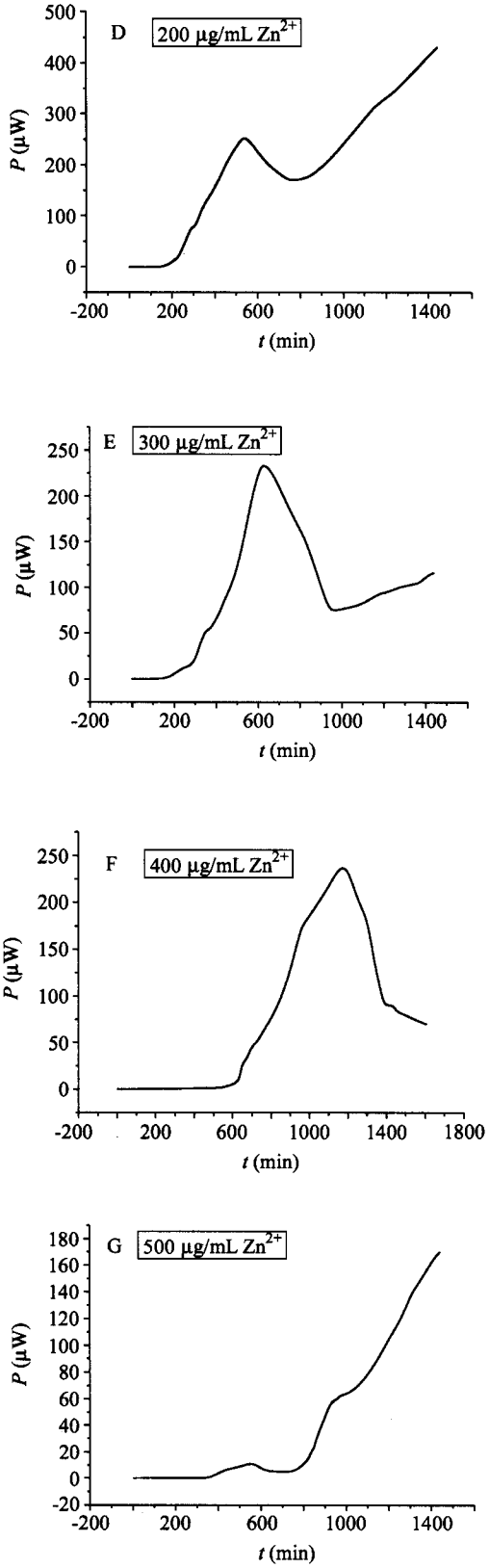


Fig. 2 Power-time curves of *Bacillus thuringiensis* at different concentrations of Zn^{2+}.

Calculation of the growth rate constant of Bacillus thuringiensis

In the log phase of growth, the cell growth is an exponential.[15,16] If the cell number is n_0 at time 0, and n_t at time t,

$$n_t = n_0 \exp(kt) \quad (1)$$

where k is the growth rate constant. If the power output of each cell is P_w, then

$$n_t P_w = n_0 P_w \exp(kt) \quad (2)$$

If the heat output power is P_0 at time 0, and P_t at time t, then

$P_0 = n_0 P_w$ and $P_t = n_t P_w$, giving

$$P_t = P_0 \exp(kt) \text{ or } \ln P_t = \ln P_0 + kt \quad (3)$$

The growth thermogenic curves of the log phase correspond to Eq. (3). So, using the data $\ln P_t$ and t taken from the curves to fit a linear equation, the growth rate constant (k) was obtained, as shown in Table 1.

Relationship between k and concentration (c)

From Table 1, it is clear that Zn^{2+} at low concentration (0—50 μg/mL) can promote the growth of *B. thuringiensis*, and Zn^{2+} at high concentration (50—500 μg/mL) is able to inhibit *B. thuringiensis* growth. In the concentration range of 100—600 μg/mL, there is not linear relationship between k and c, which was shown in Fig. 3.

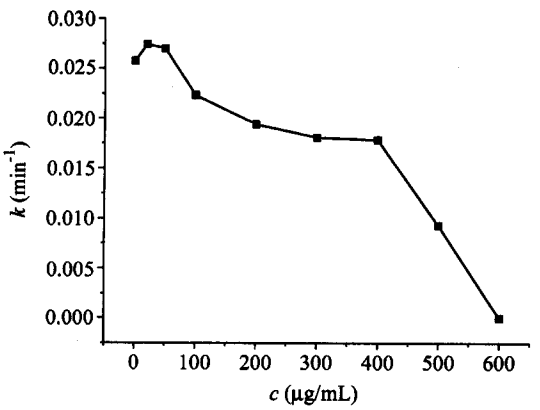


Fig. 3 Relationship between the growth rate constant (k) and c (0—600 μg/mL).

Table 1 Thermokinetic equation for *Bacillus thuringiensis* growth in log phase at different concentrations of Zn^{2+} at 28 ℃

c (μg·mL^{-1})	ln P—t	k (min^{-1})	R
0	$\ln P = -1.66182 + 0.02576t$	0.02576	0.99946
20	$\ln P = -0.95089 + 0.02700t$	0.02745	0.99798
50	$\ln P = -1.78418 + 0.02745t$	0.02700	0.99501
100	$\ln P = -0.53743 + 0.02232t$	0.02232	0.99559
200	$\ln P = -9.44782 + 0.01941t$	0.01941	0.99930
300	$\ln P = -2.31579 + 0.01808t$	0.01808	0.99691
400	$\ln P = -9.24432 + 0.01721t$	0.01721	0.99916
500	$\ln P = -4.69241 + 0.00931t$	0.00931	0.99724
600	-	0	-

Inhibitory ratios and half inhibitory concentrations

High concentration of $ZnCl_2$ will inhibit *B. thuringiensis* growth, and the growth rate constant will decrease. Therefore, the inhibitory ratio (I) can be defined as:

$$I = [(k_0 - k_c)/k_0] \times 100\% \quad (4)$$

where k_0 is the control rate constant, and k_c is the rate constant for *B. thurigiensis* growth inhibited by an inhibitor with a concentration of c. The values of I were shown in Table 2. When the inhibitory ratio I is equal to 50%, the corresponding concentration of inhibitor is called the half inhibitory concentration (IC_{50}). IC_{50} can be regarded as the inhibiting concentration of causing a 50% decrease of the growth rate constant.

Relationship of Q_{total} and c

The total heat output (Q_{total}) in 23 h is decreased with the increase of concentration of Zn^{2+} (c). Furthermore, the relationship between Q_{total} and c is linear. The linear relationship of Q_{total} and c can be obtained as:

$$Q_{total} = 26.66018 - 0.04984c, \quad R = -0.9852 \quad (5)$$

It was shown in Fig. 4.

Relationship of P_{max} and c

The relationship of the maximum power in the log phase ($P_{log-max}$) and the concentration of Zn^{2+} (c) was shown in Fig. 5, which is not linear. However, from the curve in Fig. 5,

Table 2 Experimental result of effects of Zn^{2+} on *B. thuringiensis* growth

c ($\mu g \cdot mL^{-1}$)	k (min^{-1})	I (%)	t_G (min)	Q_{total} (J)	$P_{log\text{-}max}$ (μW)	$t_{log\text{-}max}$ (min)
0	0.02576	-	26.91	16.23		
20	0.02700	-6.56	25.67	27.03	79	197.5
50	0.02745	-4.81	25.25	25.08	82.5	235.5
100	0.02232	13.35	31.05	20.38	100	241
200	0.01941	24.65	35.71	17.14	75	289
300	0.01808	29.81	38.34	8.23	52.5	350
400	0.01721	33.19	40.28	6.87	47.5	696
500	0.00931	63.86	74.45	3.65	31.5	801
600	0	100				

the low concentration and the high concentration of Zn^{2+} have different biological effect on *B. thuringiensis* growth.

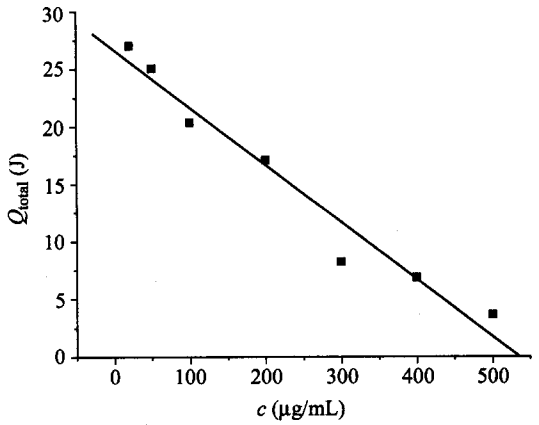


Fig. 4 Relationship between the total heat output (Q_{total}) in 23 h and c.

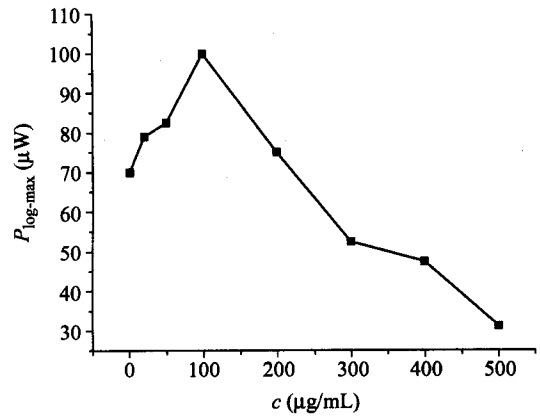


Fig. 5 Relationship between the maximum power in the log phase ($P_{log\text{-}max}$) and c.

Relationship of I and c

According to Fig. 6, the inhibitory ratio (I) is increased with the increase of concentration of Zn^{2+}, which is not linear. From Fig. 6, it can be obtained directly that IC_{50} is about 451 $\mu g/mL$.

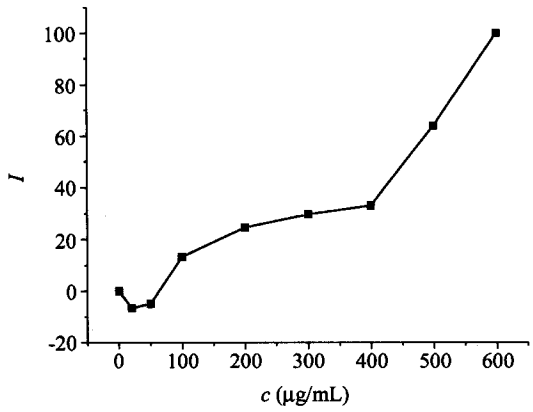


Fig. 6 Relationship between the inhibitory ratio (I) and c.

Relationship of t_{max} and c

The curve in Fig. 7 demonstrated the relationship between time of the peak of maximum power (t_{max}) and the concentration of Zn^{2+} (c). It is clear that the addition of high concentration of Zn^{2+} delays t_{max}.

Discussion

Microcalorimetry can provide both thermodynamic and kinetic information. Calorimetry can enhance the accuracy of the determination of the physiological activity of the cultures. The calorimetric determination of toxicity re-

sults in lower as well as higher values of standard data within the range of variances of toxicity determinations in different laboratory by use of the same one standard method.[8]

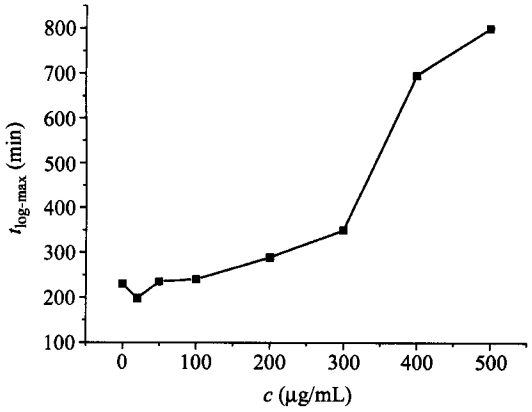


Fig. 7 Relationship between the time of peak of maximum power in the log phase ($t_{log\text{-}max}$) and c.

Direct microcalorimetry for continuous cultures is a fast response technique to determine the toxic properties of chemicals and seems to be qualified for a feed forward control strategy within biological sewage treatment.[8] Thus, microcalorimetry could be helpful in safeguarding of our environment by improving the operational safety of wastewater treatment plants.

In this study of the biologic effects of Zn^{2+} on *B. thurigiensis* growth, low concentration of Zn^{2+} has stimulating action on its growth, but high concentration of Zn^{2+} has inhibitory action on its growth. When the concentration of Zn^{2+} reaches 600 μg/mL, *B. thurigensis* will not be growth and the growth has been inhibited completely. At low concentration, Zn^{2+} is made use of the cells and protects their integrated property. So it can make the cells have appropriate flow quality and is beneficial to the synthesis of DNA and RNA in the cells, so that it is important to preserve the regular growth and the biological activity of the cells. While at the high concentration, Zn^{2+} has inhibitory action on growth. It might be explained that Zn^{2+} acts on the mercapto of the biological macromolecule and makes the proteins wreck and the proliferation is rejected. The growth rate of the cells decreases. Factors that determine the characteristics of a dose-response curve are the toxicant's mode of action in cells, its number of target sites, and its affinity for those target sites. Zn^{2+} maybe combined with sulphydryl groups on membrane proteins in all the cellular levels, resulting in cross-linking and inactivation. This changes cell membrane permeability and disrupts transport of nutrients and waste across the membrane. The toxicity of a toxicant for cells depends on its oxidation state, speciation, and the stability and solubility of its compounds. Some studied results showed a correlation between toxicity and sulphydryl affinity and it is suggested that the cross-linking of membrane proteins is a major factor in the toxic effects of materials.[17,18]

Calorimetry has been proved to be a useful tool for measuring the energy flow in natural samples. It is adaptable to toxicity studies in any type of cells. The advantage of calorimetry is that it measures the total thermal energy flow, and calorimetry in this respect is its non-specificity. By combining calorimetry with other specific methods, several different and important goals might be reached in studying the energy flow in natural environments.[18]

New methods and approaches are needed in toxicity studies and for the development of toxicity test systems, several of the presentations show potential application of calorimetry combined with other methods to determine the influence of toxicants or of eutrophication on different ecosystems.

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