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Action of palmatine on *Tetrahymena thermophila* BF_5 growth investigated by microcalorimetry

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ABSTRACT

Using a thermal activity monitor (TAM) air isothermal microcalorimeter with ampoule mode, the thermogenic curves of the metabolism of *Tetrahymena thermophila* BF_5 growth at 28 °C were obtained and the action of palmatine on it was investigated. Meanwhile, the biomass change during the process of *T. thermophila* BF_5 growth coexisted with palmatine was studied by a haemacytometer. The results showed that a low concentration (50 μg/mL) of palmatine began to inhibit the growth of *T. thermophila* BF_5, and when the concentration of palmatine reached 600 μg/mL, *T. thermophila* BF_5 could not grow at all. The relationship between the growth rate constant (k) and the concentration c was almost linear with the correlation coefficient of 0.9957, showing the strong toxic action of palmatine on *T. thermophila* BF_5 growth. The biomass during *T. thermophila* BF_5 growth decreased obviously by the addition of palmatine at different concentrations. The investigation of biomass agreed well with the results obtained by means of microcalorimetry.

1. Introduction

Ciliates were widely distributed in different natural environments. *Tetrahymena thermophila* was a freshwater ciliate belonging to the class of oligohymenophorea, subclass Hymenostomia, order Hymenostomatida, suborder *Tetrahymenina* [1]. The ciliate *T. thermophila* was a eukaryotic unicellular microorganism, which has many useful features for experimental toxicological research [2]. As an important experimental living thing, it was sensitive to the toxic substance in environment and has been selected as a standard living for the toxic detection, apoptosis and water examination [3–5]. The growth of *T. thermophila* might indicate the status of aquatic environment. This feature has allowed this microorganism to be used as a pharmacological tool in different bioassay techniques to detect toxicants [6–8].

Palmatine, an isoquinoline alkaloid originally isolated from *Rhizoma coptidis*, *Cortex phellodendri*, *Radix tinosporae*, and *Enantia chlorantha*, had extensive pharmacological actions including antibacterial activity such as *Escherichia coli*, *Staphylococcus aureus*, *etc.* [9–11], anti-inflammation [12] and anti-cancer effect [13]. Though palmatine with wide pharmacological actions has been used in many fields, the toxic action of it and the toxic action investigated by microcalorimetry have not been studied and reported from then on.

As we knew, in any living system, all the metabolic processes occurred within the cells produce heat. Thus, by monitoring the heat effects with sufficiently sensitive calorimeters, the metabolic processes of living cells could be observed and the thermo-genic curves could almost reflect the information of the metabolic processes. Microcalorimetry could directly determine the biological activity of a living system and provide a continuous measurement of heat production, thereby giving much information in both qualitative and quantitative ways [14–17]. In recent years, the application of microcalorimetry in biochemistry, biophysics and environmental sciences has received increasing attention [7,18–23]. It allowed the study of biology at the molecular level as well as at the cellular level and the thermo-genic curves contained a lot of kinetic information. *T. thermophila* produced heat by growth and metabolism. Much useful information, both qualitative and quantitative, may be obtained by monitoring the heat using a microcalorimeter. By analyzing this information, the effect of toxic substance on *T. thermophila* could be studied. So, in this study, the toxic action of palmatine on *T. thermophila* BF_5 was investigated by a TAM air isothermal microcalorimeter. Simultaneously, a haemacytometer was used to determine the population density of *T. thermophila* BF_5 cells, so there was confirmation of the general results obtained by microcalorimetry.

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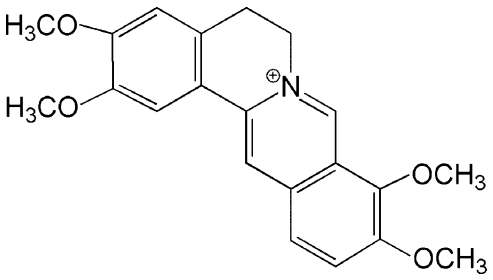

Fig. 1. The structure of palmatine.

2. Materials and methods

2.1. Instrument

T. thermophila BF_5 cells were cultivated in constant temperature incubator at 28 °C. A TAM air isothermal microcalorimeter (Thermometric AB, Sweden) was used to determine the metabolic power–time curves of *T. thermophila* BF_5 cells. This microcalorimeter is an eight-channel twin instrument and thermostated at the range of 5–60 °C, with a limit of detectability of 2 μW. For more details of the instrument, see the report of Wadsö [24]. The biomass was calculated on the haemacytometer (homemade, volume was 100 μL) with microscope imaging system (XSP-18B, Jiangnan, China).

2.2. Materials

T. thermophila BF_5 (mononuclear) was provided by the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, PR China. The culture medium was a solution (pH 7.2–7.4) containing peptone 15 g, yeast extract 5 g, glucose 1 g. It was sterilized in high-pressure steam at 121 °C for 30 min. The chemicals used in the experiments were all of analytical grade, and doubled distilled water was used to prepare all solutions. Palmatine was supplied by the National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, PR China. Its structure was given in Fig. 1.

2.3. Methods

Initially, *T. thermophila* BF_5 was cultivated in the incubator at 28 °C. Then, they were inoculated in the prepared 5 mL culture medium in a 20 mL glass ampoule, the initial population density was 4.5×10^3 cells/mL. Palmatine with different concentrations was added into each glass ampoule consequently. Then, the glass ampoules were sealed with a cap and put into the TAM air isothermal microcalorimeter. The growth process was monitored continuously and its thermo-genic curve was measured. The experiments above were all carried with aseptic technique.

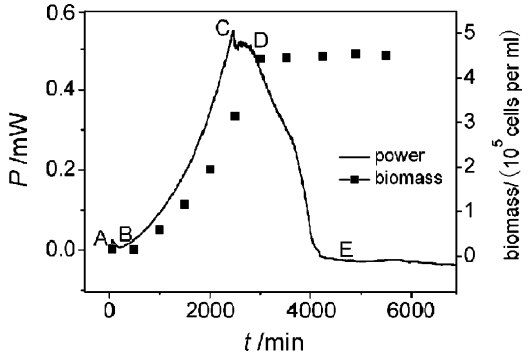

Fig. 2. Thermo-genic and biomass curves of *T. thermophila* BF_5 growth at 28 °C.

The population density of *T. thermophila* BF_5 and the influence of palmatine on biomass of *T. thermophila* BF_5 were measured with a haemacytometer. First, *T. thermophila* BF_5 cells were cultivated as above method. Then, 50 μL sample solution was extracted by using a proline single channel pipettor every 8 h into an EP pipe; then, adequate palmatine's solution diluted at an adequate proportion was added in the sample solution to kill *T. thermophila* BF_5 cells fleetly. Soon afterwards, 100 μL diluted solution was took out and dropped into the haemacytometer to calculate the biomass of *T. thermophila* BF_5.

3. Results

3.1. Thermo-genic and biomass curves of T. thermophila BF_5 growth at 28 °C

The metabolism of *T. thermophila* BF_5 growth in culture media was studied and the thermo-genic curve was recorded. The population density was counted by haemacytometer and the growth curve was measured, too. The results were shown in Fig. 2, from which we could see that the metabolic process of *T. thermophila* BF_5 could be divided into four parts: lag phase (AB), log phase (BC), stationary phase (CD) and decline phase (DE). The log phase of thermo-genic curve agreed well with that of population density. *T. thermophila* BF_5 produced heat by growth and metabolism, indicating that an increase of cell density could result in the enhancing of heat output. When *T. thermophila* BF_5 got into stationary phase and decline phase, the heat output was stopped and the biomass did not change.

3.2. Growth rate constant k and generation time t_G of T. thermophila BF_5 growth

In the log phase of growth, the cell number and heat output power was growing exponentially. So the kinetic equations are

$$P_t = P_0 \exp(kt) \quad \text{or} \quad \ln P_t = \ln P_0 + kt \tag{1}$$

The thermo-genic curves of the log phase of growth correspond to Eq. (1). Using the data $\ln P_t$ and t taken from the curves to fit a linear equation, the growth rate constant k of *T. thermophila* BF_5 growth and the correlation coefficient were obtained and shown in Table 1.

It was apparent that all of the correlation coefficients, R, were all greater than 0.9993, indicating a good reproducibility and correlation.

The log phase of biomass curves corresponded to Eq. (1), too, and the rate constant k of *T. thermophila* BF_5 growth was obtained. Consequently, the generation time (t_G), which was $(\ln 2)/k$, was also obtained. The t_G corresponded to thermo-genic curve of *T. thermophila* BF_5 was 568.2 min, and to biomass curve was 602.7 min. The values of t_G obtained from two methods were almost in agreement with each other, indicating that microcalorimetric method could record veritably the metabolism of *T. thermophila* BF_5 growth.

3.3. The effect of palmatine on T. thermophila BF_5 growth at 28 °C

The power–time curves of *T. thermophila* BF_5 growth affected by different concentrations of palmatine were shown in Fig. 3.

As could be seen from the profiles of the curves, the growth of *T. thermophila* BF_5 was significantly influenced by palmatine. Comparison to the control, the highest peak of *T. thermophila* BF_5 growth depressed gradually with the increase of the concentration of palmatine, illustrating that the metabolism of *T. thermophila* BF_5 growth was inhibited.

The k of *T. thermophila* BF_5 growth in the log phase varied along with the concentration of palmatine. The values of k degraded

Table 1
Rate constants for *T. thermophila* BF5 growth at 28 °C.

	Experiment						Mean value
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	
k (min^{-1})[a]	0.00127	0.00122	0.00119	0.00120	0.00124	0.00122	0.00122 ± 0.00008[b]
R[c]	0.9993	0.9996	0.9996	0.9996	0.9994	0.9997	0.9996
k (min^{-1})[d]	0.00115	0.00117	0.00120	0.00111	0.00114	0.00112	0.00115 ± 00009[b]
R[c]	0.9981	0.9979	0.9990	0.9963	0.9951	0.9955	0.9970

[a] Rate constant of thermo-genic curves.
[b] Mean ± S.E.
[c] Correlation coefficient.
[d] Rate constant of biomass curves.

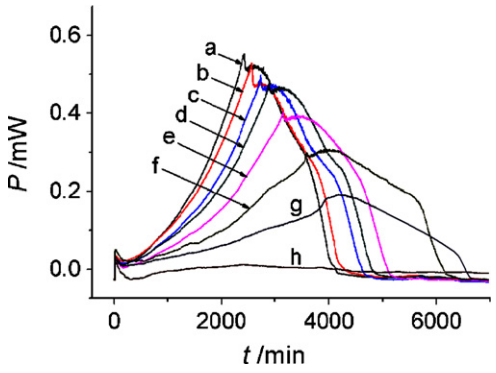

Fig. 3. The power–time curves of *T. thermophila* BF_5 growth at 28 °C affected by different concentrations of palmatine.

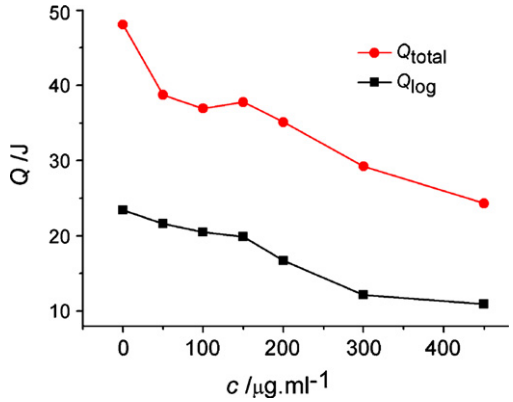

Fig. 5. Relationships between heat output in the log phase (Q_{log}), total heat output (Q_{total}) and c.

gradually with increasing of the concentration of palmatine. The relationship between k and c (shown in Fig. 4) was almost linear with the correlation coefficient of 0.9957, illustrating that palmatine of different concentrations had different actions on *T. thermophila* BF_5 growth: a low concentration (50 μg/mL) of palmatine began to inhibit the growth of *T. thermophila* BF_5. When the concentration of palmatine reached 600 μg/mL, *T. thermophila* BF_5 growth was inhibited completely: it could not grow at all. The growth inhibitory ratio could be calculated on the basis of growth rate constant. Inhibitory ratio could be defined as

$$I(\%) = \left[\frac{k_0 - k_c}{k_0}\right] \times 100\% \quad (2)$$

where k_0 was the growth rate constant of *T. thermophila* BF_5 without palmatine, k_c was the growth rate constant in the log phase of *T. thermophila* BF_5 growth inhibited at inhibitor concentration c. When the inhibitory ratio I was 50%, the corresponding concentration of inhibitor was called half inhibitory concentration, IC_{50}. IC_{50} could be regarded as the inhibition concentration of causing a 50% decrease of the growth rate constant. From the data in Table 2, we could obtain directly that IC_{50} was about 313.20 μg/mL. Meanwhile, the values of metabolic parameters of *T. thermophila* BF_5 growth changed with the increase of the concentration of palmatine. k and I increased while P_m and Q_{log}, Q_{total} were delayed with the increasing of c. The relationships between Q_{log}, Q_{total} and c were shown in Fig. 5. All these illustrated the strong toxic action of palmatine on *T. thermophila* BF_5 growth.

3.4. Biomass determination

As confirmation of the calorimetric experiments, a haemacytometer with microscope imaging system was used to evaluate the biomass change in the growth of *T. thermophila* BF_5. Through the microscope, we could see that without palmatine in the culture medium, *T. thermophila* BF_5 were active. When a low concentration of palmatine (50 μg/mL) solution was added into the medium, *T.*

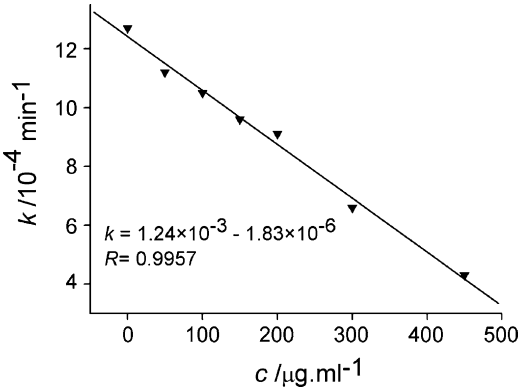

Fig. 4. Relationship between the growth rate constant (k) and c.

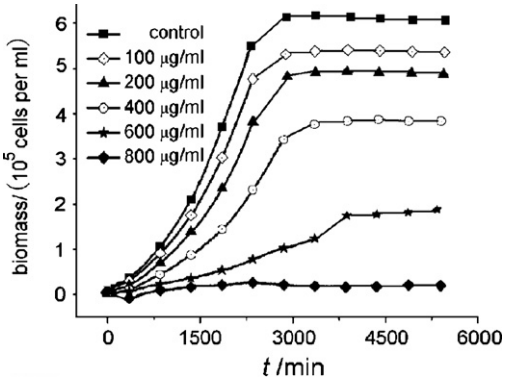

Fig. 6. Growth curves of *T. thermophila* BF_5 at 28 °C influenced by different concentrations of palmatine.

Table 2
Experimental results of effects of palmatine on *T. thermophila* BF5 growth.

c (μg/mL)	k[a] (min^{-1})	R[b]	I (%)	IC_{50} (μg/mL)	P_m (mW)	Q_{log} (J)	Q_{total} (J)
0	0.00127	0.9993	–	313.20	0.5654	23.42	4S.08
50	0.00112	0.9995	11.81		0.5374	21.63	38.72
100	0.00105	0.9996	17.32		0.5080	20.47	36.94
150	0.00096	0.9996	24.41		0.4955	19.88	37.78
200	0.00091	0.9991	28.35		0.4185	16.72	35.12
300	0.00066	0.9992	48.03		0.3224	12.14	29.24
450	0.00043	0.9995	66.14		0.2075	10.93	24.32
600	–	–	100.00		–	–	–

[a] Average of three times' experiments.
[b] Correlation coefficient.

Table 3
Enumerative results of *T. thermophila* BF5 growth affected by palmatine.

c (μg/mL)	k (min^{-1})	R[a]	I (%)	IC_{50} (μg/mL)
0	0.00115	0.9986	0	455.89
100	0.00097	0.9973	15.65	
200	0.00082	0.9977	28.70	
400	0.00067	0.9966	41.74	
600	0.00033	0.9962	71.30	
800	–	–	100.00	

[a] Correlation coefficient.

thermophila BF_5 circumrotated in the same position and began to die and rupture rapidly.

The biomass change was shown in Fig. 6. From Fig. 6, it could be concluded that cells multiplication were inhibited by the addition of palmatine, and the inhibitory action enhanced with increasing concentration of palmatine. The growth rate constant k was obtained and shown in Table 3. The half inhibitory concentration was higher than that obtained by calorimetric experiments.

4. Discussion

Bioenergetic investigations, which should be important index in the field of the evaluation of properties of harmful substances in environmental toxicology were closely related to the applicability of the direct microcalorimetric method in biology because there was scarce another method to analyze metabolic activities processes continuously and get some kinetic information, such as k, I, P_m, Q_{log}, Q_{total}, *etc.* [25]. In this study, the microcalorimetric method was successfully used to analyze the inhibitory effect of palmatine on *T. thermophila* BF_5 growth. Studying the cytotoxicity of palmatine in industrial effluent to ciliated protozoa was important and helpful in assessing the impact of drug pollution on organisms of very different complexity and position in aquatic food webs. The thermo-kinetic parameters obtained from the metabolic power–time curves could also be acted as a quantitative indicator of the toxic effect of toxicant to freshwater microbial activity [7,26].

Analyses of the power–time curves of *T. thermophila* BF_5 growth under the action of palmatine showed that the log phase became longer, the values of P_m decreased, which indicated that the normal living environment of *T. thermophila* BF_5 growth was changed and the microbes were destroyed or killed with the addition of palmatine and increasing concentrations of palmatine. When the concentration of palmatine was enough high (reached 600 μg/mL), the cells of these microbes would not grow: the growth was completely inhibited or the cells were all killed and the value of I was 100%. These results suggested that palmatine had strong capacity to inhibit the growth metabolism of *T. thermophila* BF_5 to different extents and the inhibitory capacity was concentration-depended: the toxic action of palmatine on *T. thermophila* BF_5 strengthened with increasing concentration of it.

Compared with the power–time curves and biomass curves of *T. thermophila* BF_5, the log phase of power–time curves agreed well with that of population density of the microbes. A little difference could be found (the generation time was 568.2 and 602.7 min, respectively). This owed to the distinction of the two methods self. *T. thermophila* BF_5 was intact and stable in the glass ampoule during the determination using microcalorimetry, on the other hand, biomass determination inevitably influenced the growth of *T. thermophila* BF_5. *T. thermophila* BF_5 was a kind of aerobic microbe. During the biomass determination, the culture medium need shaking up to ensure the biomass was well proportioned, so enough oxygen could be utilized by *T. thermophila* BF_5. Under this condition, *T. thermophila* BF_5 grew well and had longer generation time. And the value of IC_{50} of the toxic action of palmatine on *T. thermophila* BF_5 was different, too (313.20 and 455.89 μg/mL, respectively).

The heat output in the log phase (Q_{log}) and the total heat output (Q_{total}) has dose-relationship with the concentration of palmatine (shown in Fig. 5). By addition of palmatine into the medium, the normal living environment of *T. thermophila* BF_5 growth was changed, the microbes maybe adjust themselves to the new environment, but, some microbes which could not suit for the situation would be destroyed or killed and the heat output of the cells degraded. With the increase of concentration of palmatine, more and more microbes were killed, the log phase became longer and less and less heat output was produced. Meanwhile, the population density and biomass decreased, too.

T. thermophila BF_5 was a kind of eukaryotic unicellular microbe and used as a reference test species in toxicological tests of protozoan. And, new methods and approaches are needed for the development of toxicity test systems and studies. We anticipate that this will be one of the most important and powerful applications developed in the field of toxicology. Our experiments showed that microcalorimetry was a powerful tool for monitoring the growth process of *T. thermophila* BF_5. It provided more growth kinetic and thermodynamic information of microbe than conventional techniques and all this information was significant and useful in understanding biological processes and studying on toxic action of drug and other toxic substances.

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