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Application of microcalorimetry and chemometric analysis

Effects evaluation of angle and nail animal drugs on *Bacillus subtilis* growth

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Abstract In this study, microcalorimetry combined with chemometric analysis was used to investigate the effects of angle and nail animal valuable drugs on *Bacillus subtilis* (*B. subtilis*) growth. The power–time curves of the growth metabolism of *B. subtilis* affected by *Cornu Cervi Pantotrichum*, *Cornu Cervi Elaphi*, *Cornu Saigae Tataricae*, *cornu caprae hircus*, *Cornu Bubali*, *Squama Manis*, and *Carapax Trionycis* were determined using a thermal activity monitor (TAM) air isothermal microcalorimeter, ampoule mode, at 37 °C. By analyzing these curves and some quantitative parameters using principal component analysis, the effects of the seven animal drugs on *B. subtilis* could be quickly evaluated from the change of the two main parameters, the maximum heat-flow power P_m^2 and total heat output Q_t: *Cornu Saigae Tataricae*, *cornu caprae hircus*, *Cornu Bubali*, *Squama Manis*, and *Carapax Trionycis* inhibited the growth of *B. subtilis*, while *Cornu Cervi Pantotrichum* and *Cornu Cervi Elaphi* promoted the growth of *B. subtilis*. Further, the result of hierarchical clustering analysis showed that the drugs which promoted the growth of *B. subtilis* gathered in one cluster, the other drugs which inhibited the growth of *B. subtilis* gathered in the other cluster. All these illustrated that the internal characteristics of the seven animal drugs were different though they had similar resources and these drugs could be well clustered according the effects of them on *B. subtilis* growth with the help of chemometric methods. This study provided an useful idea of the combination of microcalorimetry and chemometric analysis for studying the effects of drugs on organisms.

Keywords Microcalorimetry · Animal drugs · *B. subtilis* · Chemometric analysis

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Introduction

In recent years, microcalorimetry has been extensively used in studying the interaction between drugs and many kinds of microorganisms [1–6]. Being an universal, integral, nondestructive, and highly sensitive tool for many biological investigations, the isothermal microcalorimetric technique directly determines the biological activity of a living system and provides a continuous measurement of heat production, which was important to evaluate the effects of extrinsic substances on the living system [7, 8]. This technique allows analysis to be performed directly on a test substance, regardless of its homogeneous or heterogeneous nature. Besides the good sensitivity, accuracy, and reproducibility, it has some peculiar advantages in new drug discovery and response for the antibacterial challenge, for example, continuous, real-time, quantitative detecting can be realized so as to obtain abundant thermokinetic/dynamic information, even the information about the mechanism of action of the antimicrobial drug [9, 10]. Thermogenic curves obtained from microcalorimetric measurements can reveal temporal details about microbial metabolism not observable by other methods [11].

Angle and nail animal drugs have got long study in China and other countries with wide therapeutical effects [12, 13]. However, the effects of some valuable angle and nail animal drugs on microbes have not been reported. In order to improve the clinical use, in this study, the effects of angle and nail animal valuable drugs including *Cornu Cervi Pantotrichum*, *Cornu Cervi Elaphi*, *Cornu Saigae Tataricae*, *cornu caprae hircus*, *Cornu Bubali*, *Squama Manis*, and *Carapax Trionycis* on *B. subtilis* growth were studied using microcalorimetry combined with chemometric analysis. The aim is to provide an useful tool of the combination of microcalorimetry and chemometric methods for investigating the effects of drugs on microbes.

Experimental

Microorganism and materials

Strain *B. subtilis* (*B. subtilis*, CMCC(B) 63501) was provided by the National Center for Medical Culture Collections, Beijing, P. R. China. It was grown in Luria-Bertani (LB) culture medium containing 2.5 g NaCl, 5 g peptone, and 2.5 g yeast extract dissolved with deionized water to 500 mL. The LB culture medium was adjusted PH to 7.0–7.2 with 1 mM NaOH and sterilized in high pressure steam at 121 °C for 30 min. The sterilized culture medium did not display the phenomenon of releasing heat according to microcalorimetry. In other words, the oxidation of the culture medium did not take place in this case. Water was purified using a Milli-Q water purification system (Milipore, Bedford, MA).

Seven animal drugs, *Cornu Cervi Pantotrichum* (S1), *Cornu Cervi Elaphi* (S2), *Cornu Saigae Tataricae* (S3), *cornu caprae hircus* (S4), *Cornu Bubali* (S5), *Squama Manis* (S6), and *Carapax Trionycis* (S7), were purchased from Beijing Tongrentang Co., Ltd., Beijing, P. R. China and were identified by Professor Xiaohe Xiao, China Military Institute of Chinese Materia Medica. All other chemicals used were of analytical grade and available locally.

Instrument

A 3114/3236 thermal activity monitor (TAM) air isothermal microcalorimeter (Thermometric AB, Sweden) was used to determine the metabolic power–time curves of *B. subtilis*. This microcalorimeter was an eight-channel twin instrument and thermostated at the range of 5–60 °C with a limit of detectability of 2 μW. The software supplied to TAM air was used to monitor and record the heat-flow power over the Peltier module when the baseline drift was less than 20 μW over 24 h. For more details of the instrument, see the report of Wadsö [14].

Preparation of sample

The seven animal drugs were grinded and decocted twice with water for 1 h. The resulting extracts were decanted, filtered, and evaporated under reduced pressure to yield the concentration of 100 mg mL^{-1}. *B. subtilis* with the density of 1×10^6 colony forming units (CFU) mL^{-1} was added into the prepared 5 mL LB culture medium in a 20 mL glass ampoule. Then, the freshly prepared drug solutions at the same concentration of 5 mg mL^{-1} were also added to the cell suspension.

Experimental procedure

This microcalorimetric measurement was performed using ampoule method at 37 °C. Eight reference ampoules containing purified water, together with another eight ampoules containing the cell suspension of *B. subtilis* and drug solutions were sealed up and put into the eight-channel calorimeter block. After about 30 min (the temperature of ampoules reached 37 °C), the power–time curves were recorded until the recorder returned to the baseline. Since the bacterial metabolic process was monitored under the isothermal and isochoric conditions, the nutrient and oxygen consumed by cells was surely limited. When the curves returned to the baseline, the experiments were finished. All data were collected continuously using the dedicated software package.

Chemometric analysis

Principal component analysis (PCA)

The PCA [15, 16] can reduce the dimensions of multivariate problems. It makes no assumptions about the underlying statistical data distribution and reduces the dimensionality of the original data set by explaining the correlation among a large number of variables in terms of a smaller number of underlying factors (PCs) without losing much information. In order to reduce the parameters from the power–time curves of *B. subtilis* growth affected by different animal drugs and find the change potency of the effects, the main parameters should be obtained. By analyzing the main parameters, the effects of these drugs could be represented quickly. So, PCA was performed on these parameters taken from the power–time curves to find out the main parameters using software of Windows SAS 8.0 (SAS, USA).

Hierarchical clustering analysis (HCA)

The HCA is a chemometric method that is used to sort samples into groups and typically illustrated by a

dendrogram [17–19]. This technique classifies samples (objects) into groups (clusters), so that each sample is similar to the others within a group but different from those in other groups with respect to a predetermined selection criterion [16]. In this part, HCA was used on the many quantitative parameters taken from the power–time curves to classify the effects of the seven animal drugs on this microbe using software of Windows SAS 8.0 (SAS, USA). The Ward's method [19] as the amalgamation rule and the squared Euclidean distance as metric were used to establish clusters.

Results and discussions

Power–time curves of *B. subtilis* growth

The metabolism of *B. subtilis* growth in LB culture medium at 37 °C was studied. The power–time curve of *B. subtilis* in the absence of any drug was recorded and displayed in Fig. 1, which could be divided into two stages: stages I and II, and four phases: the first exponential growth phase, the second exponential growth phase, a short steady phase and a decline phase [16]. The results obtained from microcalorimetry are not only in complete agreement with that of the routine microbiology method but also provide more detail information of the complex bioprocesses [20, 21].

Correspondingly, the thermogenic power–time curves of *B. subtilis* growth in the presence of different animal drugs were determined and depicted in Fig. 2. As could be seen from the profiles of these curves, the growth of *B. subtilis* was influenced by these animal drugs.

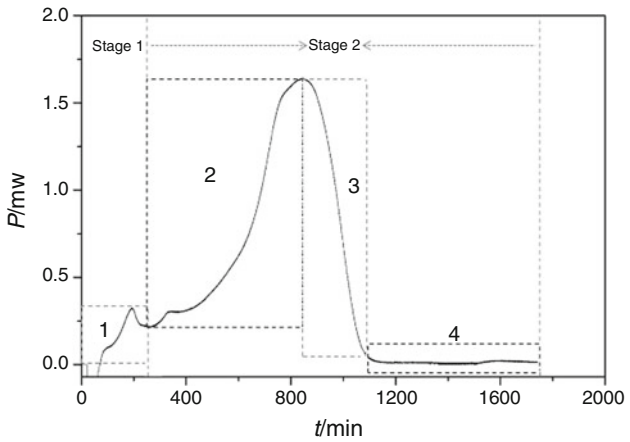

Fig. 1 Power–time curve of *B. subtilis* in the absence of any drug at 37 °C. It is a typical metabolism of *B. subtilis* culturing in LB culture medium supplemented without any drug monitored by a 3114/3236 TAM Air microcalorimeter, and can be divided into two stages: stages I and II, and four phases: (*1*) the first exponential growth phase, (*2*) the second exponential growth phase, (*3*) a short steady phase, and (*4*) a decline phase

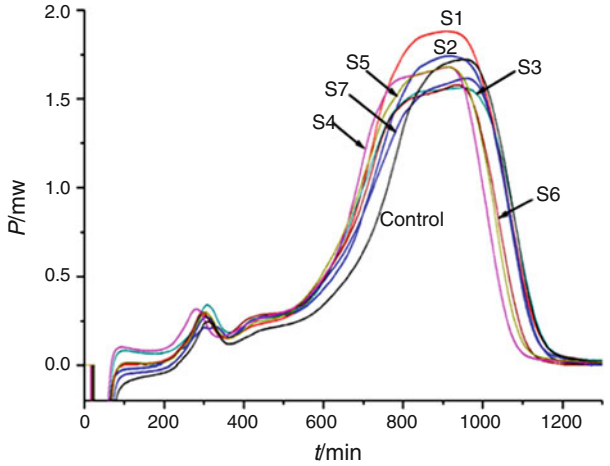

Fig. 2 Power–time curves of *B. subtilis* growth at 37 °C affected by seven animal drugs: (*control*) Blank, (*S1*) *Cornu Cervi Pantotrichum*, (*S2*) *Cornu Cervi Elaphi*, (*S3*) *Cornu Saigae Tataricae*, (*S4*) *Cornu Caprae Hircus*, (*S5*) *Cornu Bubali*, (*S6*) *Squama Manis*, and (*S7*) *Carapax Trionycis*. The peak height and appearance time of the two peaks changed with different animal drugs compared with the control, showing that different animal drugs had varied effects on *B. subtilis* growth

Quantitative thermokinetic parameters for *B. subtilis* growth

As depicted in Fig. 1, cell growth was exponential during the exponential growth phases. If P_0 and P_t are the heat flow powers at time = 0 and time = t, respectively, then: $P_t = P_0 \exp(kt)$ or $\mathrm{Ln}\, P_t = \mathrm{Ln}\, P_0 + kt$.

The growth rate constant (k_1 and k_2) for the first and second exponential growth phase can be obtained from the raised part of the first and second peak by plotting a linear curve of $\mathrm{Ln}\, P_t$ against t. Then, other metabolic thermokinetic parameters, such as the maximum heat-flow powers for the first and second exponential phase P^1_{m}, P^2_{m}, the appearance times t^1_{m}, t^2_{m} for P^1_{m}, P^2_{m}, the heat output $Q_{\mathrm{sta},1}$, $Q_{\mathrm{sta},2}$ for the first and second exponential phase, and total heat output Q_{t} for all the metabolic progress from the power–time curves of *B. subtilis* growth affected by seven animal drugs were obtained and listed in Table 1.

Effect of different animal drugs on *B. subtilis* growth

Evaluation by quantitative thermokinetic parameters

The power–time curves of *B. subtilis* growth at 37 °C affected by different animal drugs are shown in Fig. 2. The values of nine quantitative thermokinetic parameters of *B. subtilis* growth had irregular change trends (increasing or decreasing) as shown in Table 1. This phenomenon made it difficult to objectively and exactly evaluate the effect of

Table 1 Quantitative thermokinetic parameters for *B. subtilis* growth at 37 °C affected by seven animal drugs

Samples	k_1/min^{-1}	R^a	t_m^1/min	P_m^1/mW	k_2/min^{-1}	R^a	t_m^2/min	P_m^2/mW	$Q_{sta,1}$/J	$Q_{sta,2}$/J	Q_t/J
Control	0.02574	0.9964	308.0	0.248	0.00757	0.9996	950.0	1.724	1.37	40.72	42.09
S1	0.02273	0.9986	296.9	0.285	0.00784	0.9980	904.6	1.883	1.60	46.30	47.90
S2	0.01752	0.9939	328.4	0.222	0.00670	0.9997	916.2	1.745	2.03	42.47	44.50
S3	0.01881	0.9991	306.6	0.344	0.00594	0.9994	952.1	1.566	1.55	37.88	39.43
S4	0.01893	0.9981	280.9	0.319	0.00729	0.9962	911.4	1.683	1.83	39.35	41.17
S5	0.02270	0.9967	304.3	0.304	0.00602	0.9992	905.6	1.683	1.61	39.90	41.51
S6	0.02388	0.9988	296.3	0.300	0.00721	0.9998	936.4	1.581	1.55	38.88	40.43
S7	0.02537	0.9965	305.4	0.276	0.00524	0.9987	953.1	1.618	1.37	39.72	41.09

[a] Correlation coefficient

these animal drugs on *B. subtilis* growth. So, it was necessary to use suitable chemometric methods in this evaluation.

Chemometric analysis

PCA

The PCA was performed on nine quantitative parameters including k_1, t_m^1, P_m^1, k_2, t_m^2, P_m^2, $Q_{sta,1}$, $Q_{sta,2}$, and Q_t. On the basis of eigenvalues >1, two principal components (PC1 and PC2) accounting for 93.21% of the total variance were considered significant. The corresponding equations of PC1 and PC2 were shown as:

$$\begin{aligned}\mathrm{PC1} = {} & -0.120934k_1 + 0.090658t_m^1 - 0.269823P_m^1 \\ & + 0.066437k_2 - 0.368188t_m^2 + 0.465767P_m^2 \\ & + 0.276056Q_{sta,1} + 0.453445Q_{sta,2} + 0.529059Q_t\end{aligned}$$

$$\begin{aligned}\mathrm{PC2} = {} & 0.198177k_1 + 0.285592t_m^1 - 0.389263P_m^1 \\ & - 0.057652k_2 + 0.325613t_m^2 - 0.472528P_m^2 \\ & + 0.133080Q_{sta,1} + 0.206701Q_{sta,2} \\ & + 0.564072Q_t.\end{aligned}$$

The absolute values of the coefficient before these parameters represented the contribution proportion of the parameter to PC1 and PC2. It could be seen from the equation that P_m^2 and Q_t contributed more to the two PCs than the other parameters. So, P_m^2 and Q_t were the main parameters to evaluate the effect of different animal drugs on *B. subtilis* growth. From the values of P_m^2 and Q_t in Table 1, it could be found that, compare with the control, drugs S3, S6, S7, S5, and S4 could decrease the heat-flow power P_m^2 and the total heat output Q_t, while S2 and S1 could increase P_m^2 and Q_t. All these showed that *Cornu Saigae Tataricae* (S3), *cornu caprae hircus* (S4), *Cornu Bubali* (S5), *Squama Manis* (S6), and *Carapax Trionycis* (S7), could inhibit the growth of *B. subtilis*, and *Cornu Cervi Pantotrichum* (S1) and *Cornu Cervi Elaphi* (S2) promoted the growth of *B. subtilis*. These results illustrated

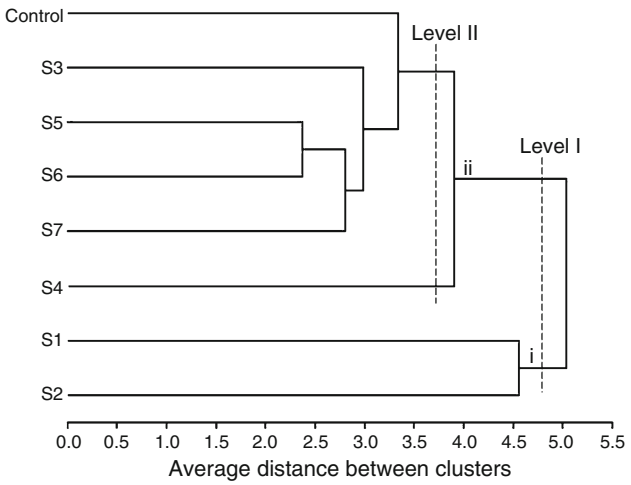

Fig. 3 Dendrogram of clustering analysis of *B. subtilis* growth affected by seven animal drugs. This dendrogram was performed by HCA using SAS 8.0 software (SAS, USA). The Ward's method as the amalgamation rule and the squared Euclidean distance as metric were used to establish clusters

that the internal characteristics of the seven animal drugs were different. In order to reveal the quality characteristics of the seven animal drugs clearly, an important statistical method HCA was introduced in the next part.

HCA

A hierarchical agglomerative clustering analysis for the nine quantitative parameters k_1, t_m^1, P_m^1, k_2, t_m^2, P_m^2, $Q_{sta,1}$, $Q_{sta,2}$, and Q_t taken from the power–time curves was performed and the results of HCA are shown in Fig. 3, from which the quality characteristics were revealed more clearly. Supposing an appropriate distance level (level I) chosen, the samples could be classified into two quality clusters. S1 (*Cornu Cervi Pantotrichum*) and S2 (*Cornu Cervi Elaphi*), which had promotion effects on *B. subtilis* growth consisted of cluster i, while the other drugs *Cornu Saigae Tataricae* (S3), *cornu caprae hircus* (S4), *Cornu*

Bubali (S5), *Squama Manis* (S6), and *Carapax Trionycis* (S7), which had inhibition effects on *B. subtilis* growth consisted of cluster ii. These showed that the drugs in cluster i had similar internal characteristics, which were different from the drugs in cluster ii. Further supposing an appropriate distance level (level II) chosen, S4 (*cornu caprae hircus*) separated as one cluster, showing that the internal characteristic of *cornu caprae hircus* was different from the drugs which in cluster ii all had inhibition effects on *B. subtilis* growth.

Conclusions

Microcalorimetry appears to be a convenient and easy technique for measuring metabolic processes through heat production in complex samples such as living cells. In this study, microcalorimetry was successfully used to evaluate the effects of seven animal drugs on *B. subtilis* growth. Combined with chemometric analysis PCA and HCA, it could find that the effects of these animal drugs were different. *Cornu Cervi Pantotrichum* and *Cornu Cervi Elaphi* could promote the growth of *B. subtilis*, while *Cornu Saigae Tataricae*, *cornu caprae hircus*, *Cornu Bubali*, *Squama Manis*, and *Carapax Trionycis* inhibited the growth of *B. subtilis*. These results showed that the internal characteristics of the seven animal drugs were different though they had similar resources. The drugs could be well clustered according the effects of them on *B. subtilis* growth with the help of chemometric methods.

Our results would provide some references for the use of these animal drugs in clinic. Further study should be focused on investigating the effects of these drugs on more microbes and the mechanism of action. This study also provided an important idea of the combination of microcalorimetry and chemometric analysis for studying the effects of other drugs on living cells. In conclusion, microcalorimetric investigations on microorganisms are possible and promising. It is believed that microcalorimetry is an useful and accurate system for studying the detailed effects of drugs on microorganisms to warrant the clinical use of these drugs.

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