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A MICROCALORIMETRIC METHOD FOR STUDYING *HALOBACTERIUM HALOBIUM*

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Abstract

The application of a microcalorimetric method to the study of extremophiles is described briefly. Using the LKB 2277 Bioactivity Monitor, the growth thermogenic curves of three strains of *Halobacterium halobium* were determined at 37°C, and compared with the spectrophotometric curves. Then the suitable growth thermokinetic equation was established based on the characteristics of growth thermogenic curves. By using cycle-flow method, all of the growth thermogenic curves of *H. halobium* strains displayed a brief lag phase before the onset of exponential growth when they were cultured in Halo-2 medium.

Keywords: extremophiles, *Halobacterium halobium*, microcalorimetry, thermokinetics

Introduction

All chemical, physical and biological processes are correlated with heat. Calorimetry is therefore a very powerful technique for the detection and quantification of unknown or unexpected events being parts of complex systems [1]. Although the microcalorimetric method lacks specific property, the research samples have their unique characteristics. So, new results can be obtained only by this method. In recent years, microcalorimeters have been used in a number of microbiological applications, such as bacteria [2], yeast [3] and other mixed microbial systems [4]. As modern microcalorimeters can measure heat output powers well below 1 μW, it is clear that microcalorimetry is a sensitive technique for studying microbial activity.

Microorganisms are able to colonize many extreme environments and can display active growth under such conditions [5, 6]. Based on their respective original habitats, four parameters are commonly used to classify these so-called 'extremophiles': (1) high and low temperatures, defining thermophiles and psychrophiles, respectively; (2) high and low pH values, defining alkaliphiles and acidophiles, respectively; (3) high salt concentrations, defining halophiles; (4) high pressure, defining barophiles [7]. Extremophiles have unique genomes, special physiological mechanisms, and unique metabolic processes. They provide us with new insights into the origin of life, phylogeny, and bio-

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diversity. They also open up a new concept of life behavior, as well as give a new chance to develop biotechniques [8]. Extremophiles have become the subject of considerable scientific research in recent times, however it is very difficult to culture and manipulate these organisms by the conventional microbiological methods. At the same time, their slower growth makes them difficult to study. *Halobacterium halobium* is one kind of halophiles. *H. halobium* requires a concentration of 4.3 mol L^{-1} NaCl for growth in culture medium. Concentrations of 0.1–0.5 mol L^{-1} Mg^{2+} and 1.3–2.5 mol L^{-1} K^+ are also required for optimal growth [9]. The popular view is that *H. halobium* accumulates inorganic salts in the cytoplasm to cope with osmotic stress. K^+ rather than Na^+ is the dominant intracellar cation, and Cl^- the dominant anion [10].

In this investigation, we studied the growth metabolism of three strains of *H. halobium* (R1, J7 and F9) by the microcalorimetric method. We obtained their growth power-time curves, and discovered that a short lag phase preceding the exponential growth in Halo-2 medium. When *H. halobium* R1 was cultured in Halo-8 medium, the lag period was not observed. This is the report of microcalorimetry applied to the study of *H. halobium* and describes an important technique for research on other extremophiles.

Experimental

Materials

Strains

Halobacterium halobium R1 (no plasmid), J7 (carrying plasmid pHH205) and F9 were provided by the China Center for Type Culture Collections, Wuhan University, P. R. China. F9 strain, carrying plasmid pHH205 from J7, was a fusion strain between J7 and R1 strain. It was obtained by using killed-parents method [11].

Culture medium

Halo-2 medium: NaCl 250 g, $MgCl_2 \cdot 6H_2O$ 30 g, Yeast extract 2 g, Lactalbumin hydrolysate 2.5 g, H_2O 1000 mL, natural pH.

Halo-8 medium: NaCl 250 g, $MgCl_2 \cdot 6H_2O$ 30 g, K_2SO_4 2 g, Yeast extract 2 g, Lactalbumin hydrolysate 2.5 g, H_2O 1000 mL, natural pH.

Calorimeter

The LKB 2277 Bioactivity Monitor, which is a type of heat-flow microcalorimeter, was used to determine the growth metabolism of *H. halobium*. The performance of this instrument and the details of its construction have been previously described [12].

Experimental procedure

Growth power-time curves of *H. halobium*

The temperature of the detection system was controlled at 37°C.

30 mL sterilized medium was pumped through the LKB2277 Bioactivity Monitor system at a flow rate of 30 mL h^{-1} with constant stirring, initially added 30 μL full growth culture containing $6.3 \cdot 10^6$ cells.

Growth curves of *H. halobium*

Following cultured for three days at 37°C, 0.5 mL of full growth culture was aseptically transferred into 50 mL Halo-2 medium in a 250 mL flask. The inoculated flask was shaken vigorously (200 rpm) at 37°C. Measuring the optical density at 550 nm at various times, and plotting optical density *vs*. time obtained growth curves.

Growth thermokinetics

Cui *et al*. [13, 14] proposed an ecological nutrition kinetics model according ecological characteristics from adsorption of chemical kinetics, the classical exponential model and logistic model could be revised as [15]:

$$dN/dt=kN(1-N/N_m)/(1-N/N'_m) \quad (1)$$

N is the cell number at time t, N_m is the largest cell number in the growth phase, N'_m is the cell number when all nutrient in culture medium has been used up.

If the power output of each cell is W, then $N=P/W$

$$dP/dt=kP(1-P/P_m)/(1-P/P'_m) \quad (2)$$

If $\alpha=1/P_m$, $\beta=1/P'_m$, then

$$dP/dt=kP(1-\alpha P)/(1-\beta P) \quad (3)$$

$$[(1-\beta P)/(P(1-\alpha P))]dP=kdt$$

If $r=\beta/\alpha$, giving

$$(1/P)dP+[(r-1)/(1-\alpha P)]d(1-\alpha P)=kdt$$

$$[\ln P+\ln(1-\alpha P)^{r-1}]-[\ln P_0+\ln(1-\alpha P_0)^{r-1}]=kt \quad (4)$$

So

$$\ln[P(1-P/P_m)^{r-1}]=\ln[P_0(1-P_0/P_m)^{r-1}]+kt \quad (5)$$

Equation (5) is abnormal 's' growth thermokinetic equation.

If $r=1$, $\alpha=\beta$, Eq. (2) becomes

$$dP/dt=kP \quad (6)$$

It is an exponential growth equation.

If $r=0$, $\beta=0$, Eq. (2) becomes

$$dP/dt=kP(1-P/P_m) \quad (7)$$

It is a logistic growth equation.

Results

Growth curves of Halobacterium halobium

The growth power-time curves of *H. halobium* strains at 37°C are shown in Fig. 1.

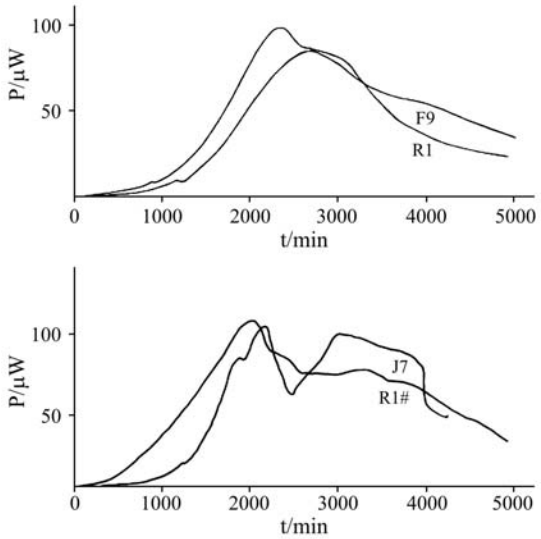

Fig. 1 The thermogenic power-time curves of *Halobacterium halobium* strains at 37°C. R1#–R1 strain was cultured in Halo-8 medium; the curve is smooth. The other strains were cultured in Halo-2 medium; the thermogenic curves all display a little shoulder of approximately 1 h duration before the exponential growth

When *H. halobium* strains were cultured in Halo-2 medium, the thermogenic curves all display a short lag phase of approximately 1 h duration before the exponential growth. When strain R1 was cultured in Halo-8 medium (Halo-2 medium with 0.2% K_2SO_4), the short lag phase was not observed.

The growth curves at 37°C are shown in Fig. 2.

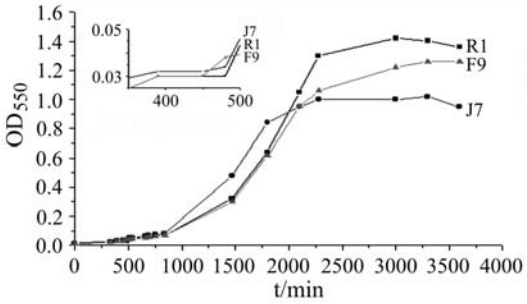

Fig. 2 The growth curves of *Halobacterium halobium* strains at 37°C. R1, F9, J7 strains were cultured in Halo-2 medium. The small graph is the amplified growth curves graph of *H. halobium* between 350 and 500 min

The growth curves show a remarkable stationary phase before exponential phase, by detecting spectroscopy values at OD_{550} once for thirty min between 7 and 12 h.

Growth thermokinetic equation and generation time

The thermogenic curves of the growth phase correspond to Eq. (5). So making use of the data P_t and t taken from the curves to fit Eq. (5), r runs in the range of 0–1, recycling calculation and linear regression, r value were obtained when the best correlationship and the optimum correlation coefficient (R) were achieved. Therefore, the growth thermokinetic equations of *H. halobium* R1, J7 and F9 strains were obtained. The results are shown in Tables 1 and 2.

Table 1 The data of calculation and regression analysis on *Halobacterium halobium*

H. halobium	*r*	*R*	*H. halobium*	*r*	*R*
	0.000	0.95322		0.000	0.94634
R1 ①	0.817	0.99826*	J7	0.897	0.99981*
	1.000	0.98402		1.000	0.99399
	0.000	0.95199		0.000	0.96354
R1 ②	0.828	0.99868*	F9	0.741	0.99499*
	1.000	0.98585		1.000	0.96103
	0.000	0.96144		0.000	0.98106
R1 ③	0.801	0.99939*	R1#	0.659	0.99587*
	1.000	0.97821		1.000	0.97935

*Optimum correlation coefficient; R1# – R1 strain was cultured in Halo-8 medium. The other strains were cultured in Halo-2 medium, R1 were detected three times; r – the efficiency of nutrient utilization by an organism; R – correlation coefficient

Table 2 The thermokinetic equation of growth of *Halobacterium halobium*

H. halobium	*T*/°C	Thermokinetic equation	*R*	$1-P_m/P'_m$
R1 ①	37	$\ln[P(1-P/98.1)^{-0.183}]=-1.9529+2.29\ 10^{-3}t$	0.99826	0.183
R1 ②	37	$\ln[P(1-P/98.1)^{-0.172}]=0.09631+2.28\ 10^{-3}t$	0.99868	0.172
R1 ③	37	$\ln[P(1-P/96.5)^{-0.199}]=-0.6419+2.52\ 10^{-3}t$	0.99939	0.199
J7	37	$\ln[P(1-P/77.6)^{-0.103}]=-1.8119+3.42\ 10^{-3}t$	0.99981	0.103
F9	37	$\ln[P(1-P/84.5)^{-0.259}]=-0.5762+2.37\ 10^{-3}t$	0.99499	0.259
R1#	37	$\ln[P(1-P/108.0)^{-0.341}]=1.5715+2.01\ 10^{-3}t$	0.99587	0.341

R1# – R1 strain was cultured in Halo-8 medium. The other strains were cultured in Halo-2 medium, R1 were detected three times; R – Optimum correlation coefficient

The growth rate may also be described in terms of the time required for the cell population to double in number. This is the generation time (T_G). According to $T_G=(\ln 2)/k$ [12], we can calculate T_G values from k values of the three strains in Table 2. When strains were cultured in Halo-2 medium, the T_G values were 294, 203 and 293 min

for strains R1, J7 and F9, respectively. However, when cultured in Halo-8 medium, the generation time for R1 was 50 min longer.

Discussions

Reproducibility and growth equation

From the data obtained from *H. halobium* R1 and shown in Table 1, it is apparent that r=0.815±0.011. All of the optimum correlation coefficients are larger than 0.9950. In Table 2, k=0.0023605±0.00001105 min^{-1}, indicating a good reproducibility and correlationship. This demonstrates that good results can be obtained by the application of the microcalorimetric method to extremophiles. This lends support both for the further development of microcalorimetric research of extremophiles and for exploring the growth characterization of *H. halobium* in abnormal condition.

Whether the data were regressed according to the exponential growth Eq. (6) or the logistic growth Eq. (7), the correlation coefficients (R) all were lower than the optimum correlation coefficients, which were regressed by Eq. (5) (Table 1). This suggests that the abnormal 's' growth thermokinetic Eq. (5) is very suitable for *H. halobium* growth. The growth thermokinetic equations of three strains are shown in Table 2. From the tables the efficiency of nutrient utilization (r) of three strains is different. The efficiency of nutrient utilization of fusion strain F9 was apparently lower than that of its parent strains (R1 and J7). R1 strain could utilize nutrient more efficiently in Halo-2 medium than it did in Halo-8 medium. So 0.2% K_2SO_4 perhaps is not very suitable for R1 strain growth.

Different H. halobium strains

The T_G values showed that J7 strain grew fastest, R1 strain grew slower, F9 strain was near R1 and its growth period was prolonged. Meanwhile, the growth rate constants of *H. halobium* strains conformed to growth rates of growth curves. The growth rate constant of J7 strain was higher than that of R1 strain, J7 strain grew rapidly than R1 strain. While the two strains were fused, the growth rate of the fusion had relation with two parent strains. The growth rate constant of F9 strain was close to that of R1. Its optimum correlation coefficient was lower than that of parent strains. It is probable that the genetic characteristics of F9 strain are not the same as R1 or J7, its growth and metabolic characteristics are also changed. Its growth and thermogenic power curves are distinctly different from the two parent strains.

Growth thermogenic curves

The thermogenic curves of bacterial metabolism completely describe growth metabolism processes. Under the same conditions, the thermogenic curves of every kind of bacteria all have good reproducibility and outstanding characteristics. Once experimental factors varied, the metabolic process will be influenced, hence the bacterial metabolic thermogenic curves changed remarkable. Microcalorimetry has been useful in measuring the ef-

fects of various substances and culture conditions on organisms' metabolism [16]. Microcalorimetric studies of bacterial growth also reveal temporal details not observable by other techniques. It is more notable for those slow-growing extremophiles. When *H. halobium* strains were cultured in Halo-2 medium, the thermogenic curves all displayed a little shoulder before the logarithm phase (Fig. 1). The brief lag phase lasted about one hour. While the strain was cultured in Halo-8 medium (only complemented 0.2% K_2SO_4 into Halo-2 medium), the little shoulder has not been observed. This phenomenon was difficult to discover using the conventional microbiological techniques. The growth curves did have the little stationary phase before logarithm phase when detecting their values of OD_{550} once for thirty min between 7 and 12 h (Fig. 2). Some papers [10, 17] reported that in the extreme halophiles, the differences between internal and external Na^+ concentration exceeded 2 mol L^{-1}. While K^+ concentration in vivo was 4 to 5 mol L^{-1}, higher than that in the medium. But K^+ is so minute in Halo-2 medium that the strain stabilizes for a small period of time before the start of exponential growth. During this period, perhaps the strains synthesized organic osmotic comptabile solutes such as glycerol, glycine betaine, ectoine instead of K^+ to maintain osmotic balance. Or the strains use other unknown strategies of osmoadaptation in Halo-2 medium. While R1 strain was cultured in Halo-8 medium, the growth power-time curve did not show the little shoulder. The R1 strain can pump Na^+ out of the cell by the Na^+/H^+ antiporter transporter system, and K^+ probably enter the cell passively in response to the membrane potential [10]. We can make the conclusion from the above analysis, although *H. halobium* strains live in high salt environment, grow slowly, their growth thermogenic curves are in accordance with the growth curves, can represent growth and metabolic characteristics and reflect variance of physiological and biochemical characteristics.

The genome of *Halobacterium* is notable for the presence of dynamic replicons and a variety of transposable elements that give rise to frequent DNA rearrangements [18]. The complete sequence of *Halobacterium* NRC-1 genome has been reported [19]. So far, most extrachromosomal elements or plasmids were found to contain genes that are normally thought to be nonessential for cell viability. Interestingly, about 40 genes on plasmids pNRC100 and pNRC200 of *Halobacterium* NRC-1 coded for proteins likely to be essential or important for cell viability. So the plasmids in halophiles have close relation with their hosts. The thermal power-time curves of *H. halobium* J7 and R1 were apparently different (Fig. 1); the former was more complex than the latter. We know nothing about *H. halobium* J7 except that it harbors a large plasmid pHH205. Just as Professor Wadsø reported, signals from complex reaction systems can be very difficult to interpret if they are not supported by results from specific analytical measurements [20]. However, many interesting metabolic characteristic details have been obtained from the thermal power-time curves, so that we can research them further by other techniques.

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