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Effect of nisin on the growth of *Staphylococcus aureus* determined by a microcalorimetric method

Xiaohong Wang[1], Ying Liu[1], Bijun Xie[1], Xianming Shi[2], Junchu Zhou[3] and Honglin Zhang[4]

[1]Department of Food Safety and Microbiology, HuaZhong Agricultural University, Wuhan, P. R. China
[2]Department of Food Science and Technology, Shanghai Jiaotong University, Shanghai, P. R. China
[3]National Key Laboratory of Agricultural Microbiology, HuaZhong Agricultural University, Wuhan, P. R. China
[4]Department of Chemistry, Qufu Normal University, Qufu, P. R. China

A novel microcalorimetric technique based on the bacterial heat output was applied to evaluate the biological effect of nisin on the growth of *Staphylococcus aureus*. The thermogenic curves of *S. aureus* in the presence of nisin were studied by an LKB-2277 Thermal Activity Monitor. The thermokinetic parameters, such as the growth rate constant (k), the generation times (G), the inhibitory ratio (I), and the half inhibitory concentration (IC_{50}), for the growth of *S. aureus* at different nisin concentrations were determined. The relationship between the growth rate constant (k) and the concentration of nisin (c) is nearly linear, which can be modeled by the formula $k = 0.03794 - 4.005 \times 10^{-4} \times c$, with a correlation coefficient of -0.9971. Based on this model, we obtained the critical inhibitory concentration of nisin on the growth of *S. aureus* at 94.73 IU/mL. We proposed that this microcalorimetric method could be a useful tool in monitoring the biological effect of nisin on microorganisms, and providing valuable information on the study of microorganism metabolisms.

Keywords: Microcalorimetry / Nisin / *Staphylococcus aureus* / Thermochemistry / Thermogenic growth curve

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1 Introduction

Nisin, produced by *Lactococcus lactis subsp. lactis*, is a small, heat-stable protein. Classified as a lantibiotic, it is one of the best-known bacteriocin [1]. Nisin primarily inhibits Gram-positive bacteria [2], either alone or in combination with other compounds. It is widely used as a preservative in food industry including dairy, canned food, brewing, and alcohol fermentation. Nisin is generally regarded as safe (GRAS) substance [3] and is widely used in over 50 countries [4]. The biotechnological applications of nisin for the control of food-borne pathogenic bacteria have increased with the discovery of its inhibitory effect on various food-borne bacteria, such as *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* [5, 6]. The mechanism of nisin [7–10], factors influencing its activity [11, 12], and its application as food preservative [13] have been studied extensively. Research on the microcalorimetric changes during the treatment of microorganisms with antimicrobials, such as nisin, is scarce.

The microcalorimetric technique is one of the most important methods for thermodynamic studies. In some living systems, the various metabolic events occurring within the cells are heat-producing reactions. Thus, by monitoring the heat effect of the growing cells with a sufficiently sensitive microcalorimeter, the thermogenic curve obtained could reflect time-dependent changes in the growth pattern of the cells tested [14]. It has been widely applied to determine the functionary effects of drugs on biological subjects, such as microorganisms, cultured tissue cells, tissue pieces, and biological macromolecular because of its high sensitivity and full automation [15–17].

The growth thermograms of bacteria can be determined by the microcalorimetric method [18]. These perfect thermogram curves reflect the changes of bacterial growth patterns (including the lag phase of growth, log growth, stationary phase, and the decline phase of growth). Based on the thermogenic curve, both thermodynamic and kinetic information about the characterization of the microbial growth under a certain set of growth conditions can be observed. Since the thermogenic curve provides useful information in

Correspondence: Prof. Bijun Xie, Department of Food Safety and Microbiology, HuaZhong Agricultural University, Wuhan 430070, P. R. China
E-mail: bijunxie@sina.com (Bijun Xie) wxh@mai.hzau.edu.cn (Xiaohong Wang)
Fax: +86-27-87282966

Abbreviation: LB, Luria-Bertani medium

both qualitative and quantitative ways [19], it helps us to understand the thermodynamical rules of bacteria during the metabolic process. During the past 30 years, microcalorimetry has been used extensively to investigate drugs and the microbial cell interaction [20, 21]. Liu *et al.* [22] demonstrated that microcalorimetric studies of bacterial growth revealed temporal details not observable by other techniques.

In this study, the power-time curves produced by *S. aureus* ACCC10041 in the presence of nisin at different concentrations were studied by an LKB-2277 Thermal Activity Monitor. Some principal parameters for microbial growth, such as the growth rate constant (k) and the generation time (G), were calculated. The linear model between growth rate and nisin concentrations was constructed and the critical inhibitory concentration of nisin was determined from the model. Our studies show that the automatic microcalorimetric method is a powerful tool to study microbial growth in the presence of bacteriocins, such as nisin.

2 Materials and methods

2.1 Bacterial strain and culture conditions

Staphylococcus aureus ACCC10041, provided by the Agricultural Culture Collection of China, was used throughout this study. The stock culture was maintained on nutrient agar slants at 4°C and transferred monthly onto a fresh medium. The stock culture was transferred to 10 mL Luria-Bertani (LB) medium and incubated for 16 – 18 h at 37°C prior to the microcalorimetry experiment. LB medium consists of 0.5% NaCl, 1% Bacto tryptone, and 0.5% Bacto yeast-extract, pH = 7.2. The medium was sterilized by autoclaving for 20 min at 0.1 MPa.

2.2 Nisin preparation

Nisin was purchased from MP Biomedicals (Eschwege, Germany). A nisin stock solution (10^6 IU/mL) was prepared by dissolving 0.1 g nisin in 80 mL HCl (0.02 mol/L) and held at room temperature for 2 h for complete dissolution. The volume was then made up to 100 mL with HCl (0.02 mol/L) and the solution was filter-sterilized by passing through a 0.22-μm Millipore membrane and stored at –20°C.

2.3 Instrument and microcalorimetric experiment

An LKB-2277 Thermal Activity Monitor (ThermoMetric-AB, Järfälla, Sweden) was used to determine the power-time curves of bacterial growth. The microcalorimeter was thermostated at 37°C. The voltage signal was recorded by means of an LKB-2210 recorder (1000 mV range). The detection limit was 0.15 μW and the baseline stability (over a period of 24 h) was 0.2 μW. The performance of this instrument and the details of its construction have been described previously [23]. In the calorimetric experiment, the flow cell was completely cleaned and sterilized, obeying the following procedure: sterilized distilled water, 0.1 mol/L HCl, 0.1 mol/L NaOH, 75% alcohol solution, and sterilized distilled water were pumped in sequence by an LKB-2132 microperpex peristaltic pump through the cell, each for 30 min at a flow rate of 30 mL/h. Once the system was ready, the bacterial suspension at a concentration of 1×10^6 cells/mL and nisin was pumped through the calorimetric cell at a flow rate of 30 mL/h. When the flow cell (0.6 mL) was filled, the pump was stopped and power-time curves of the bacterial growth were recorded by the monitor.

3 Results and discussion

3.1 Power-time curves of *S. aureus*

The power-time curve for growth of *S. aureus* in LB medium at 37°C is shown in Fig. 1, which is matched with the thermogenic growth curve. The curve can be divided into four phases [24], that is, lag phase, log phase, stationary phase, and decline phase. In the power–time curves, the first peak represents the logarithmic phase of growth. The power of the curve correlated well with the microbial mass (data not shown).

Power-time curves for the growth of *S. aureus* at 37°C in the presence of nisin at different concentrations are shown in Fig. 2. The lag phase, which is between the start of the experiment and the ascending phase of the power-time curve, became longer with the increase of nisin concentration. The time to attain the log phase was 295 min for *S. aureus* at 60 IU/mL nisin, as compared to the normal growth

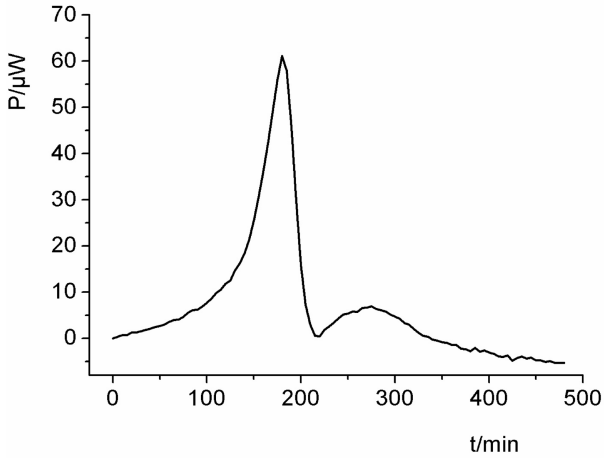

Figure 1. Power-time curve of the growth of *S. aureus* in LB at 37°C.

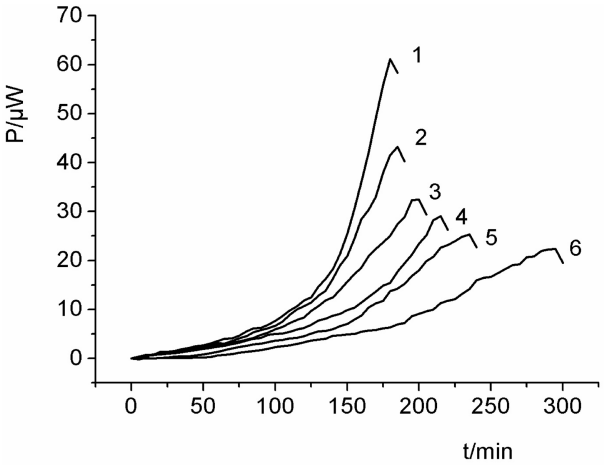

Figure 2. Power-time curves of the growth of *S. aureus* in the presence of different nisin concentrations at 37°C: (1) 0; (2) 10 IU/mL; (3) 20 IU/mL; (4) 30 IU/mL; (5) 40 IU/mL; (6) 60 IU/mL.

time of 180 min. A high concentration of nisin had a larger effect on the log phase of *S. aureus*. When the nisin concentration increased from 0 to 20 IU/mL, the log time increased from 180 to 200 min. However, when the nisin concentration was increased from 40 to 60 IU/mL, the log time was increased from 235 to 295 min. The heat output of *S. aureus*, which corresponded with the peak of the curve at log phase time, decreased as the concentration of nisin increased. The heat outputs were substantially reduced by adding 20 IU/mL nisin, as shown in curves 1 and 3. In curve 1, the heat output from normal growth of *S. aureus* is 61.12 μW. It decreased to 32.47 μW when 20 IU/mL nisin was added. At 40 IU/mL, the heat output was 25.28 μW and further decreased to 22.40 μW at 60 IU/mL nisin.

3.2 Calculation of the growth rate constant (*k*) and the generation times (*G*) of *S. aureus*

The growth curves of *S. aureus* showed that the log phase of growth obeyed the growth model of bacteria [25, 26].

In the log phase of growth, with the cell number n_0 at time 0, and n_t at time t, then

$$n_t = n_0 \times \exp(k \times t) \quad (1)$$

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

$$n_t P_w = n_0 \times P_w \times \exp(k \times t) \quad (2)$$

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

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

By selecting a series of P_t and t from the log phase of the power-time curves of *S. aureus* and using an exponential law, we calculate the growth rate constants of *S. aureus* at different concentrations of nisin, and the correlation coefficient R^2. The generation times (G) of the growth of *S. aureus*, which are (ln 2)/k, can also be obtained. The corresponding k, R^2, and G are shown in Table 1. From Table 1 we found that the generation times (G) of the growth of *S. aureus* increased with the increase of the concentrations of nisin. All correlation coefficients are higher than 0.9879, indicating a good correlation.

3.3 Relationship between the growth rate constants (*k*) and concentrations of nisin (*c*)

The values of the growth rate constants (k) in Table 1 show that different concentrations of nisin have different inhibitory effects on the growth of *S. aureus*. The growth rate constants (k) decrease with the increase of the concentrations of nisin (c). From Table 1 we can obtain the k–c equations:

$$k = 0.03794 - 4.005 \times 10^{-4} c \text{ and } R = -0.9971$$

where k is the growth rate constant of *S.aureus*, and c is the concentration of nisin.

Table 1. Growth rate constant (*k*), generation times (*G*), and inhibitory ratio (*I*) of *S. aureus* in the presence of different nisin concentrations

System	c (IU/mL)	k (min^{-1})	G (min)	R^2	I (%)
Control	0	0.03739	18.5	0.9992	–
Nisin	10	0.03354	20.7	0.9919	10.30
	20	0.03031	22.9	0.9986	18.93
	30	0.02519	27.5	0.9969	32.63
	40	0.02278	30.4	0.9881	39.07
	60	0.01320	52.5	0.9879	64.70

Figure 3 shows the relationship of the growth rate constants (k) and concentrations of nisin (c). From the k–c equation and Fig. 3 we extrapolate that when the concentration of nisin reaches 94.73 IU/mL, the growth rate constant of *S. aureus* is zero. This concentration is defined as the critical concentration of nisin (C_0), which indicates that nisin exerts complete inactivation of *S. aureus*; at this time no heat-out can be observed in the power-time curve.

3.4 Inhibitory ratios (*I*) and half inhibitory concentration (IC_{50})

The inhibitory effect of nisin at different concentrations on the growth of *S. aureus* can be compared by the inhibitory ratio. The inhibitory ratio (I) is defined as:

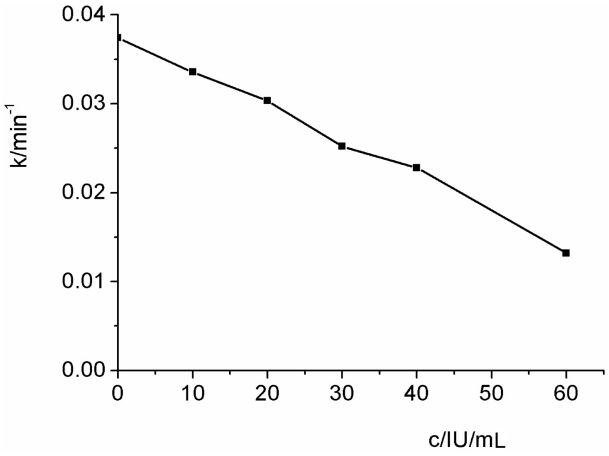

Figure 3. Plot of the growth rate constant (*k*) of *S. aureus vs.* nisin concentration (*c*).

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

where k_0 is the growth rate constant of *S. aureus* without nisin and k_c is the growth rate constant of *S. aureus* with nisin at different concentrations. The nisin concentration is called the half inhibitory concentration (IC_{50}) when the inhibitory ratio is 50%. According to the $I-k$ equation, the inhibitory ratios (I) with nisin at different concentrations can be obtained. The results are shown in Table 1; the half inhibitory concentration (IC_{50}) is 48.05 IU/mL.

4 Concluding remarks

Our results show that the growth rate constants (k) of *S. aureus* are in inverse proportion to the concentrations of nisin, and the $k-c$ equation is linear with the correlation coefficient of −0.9971. By means of the power-time curves we can estimate thermokinetic parameters, such as the growth rate constant (k), the generation times (G), the inhibitory ratio (I) and the half inhibitory concentration (IC_{50}) for the growth of *Staphylococcus aureus* in the presence of nisin. Even though the action of nisin against *S. aureus* may be strain-dependent, we believe that our studies on this thermochemical aspect are also applicable to other researches. In conclusion, microcalorimetry is a powerful tool for monitoring and controlling the growth of microorganisms, which can be very informative for understanding the biological effect of nisin on other microorganisms as well.

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