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Maximum-biomass prediction of homofermentative *Lactobacillus*

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Fed-batch and pH-controlled cultures have been widely used for industrial production of probiotics. The aim of this study was to systematically investigate the relationship between the maximum biomass of different homofermentative *Lactobacillus* and lactate accumulation, and to develop a prediction equation for the maximum biomass concentration in such cultures. The accumulation of the end products and the depletion of nutrients by various strains were evaluated. In addition, the minimum inhibitory concentrations (MICs) of acid anions for various strains at pH 7.0 were examined. The lactate concentration at the point of complete inhibition was not significantly different from the MIC of lactate for all of the strains, although the inhibition mechanism of lactate and acetate on *Lactobacillus rhamnosus* was different from the other strains which were inhibited by the osmotic pressure caused by acid anions at pH 7.0. When the lactate concentration accumulated to the MIC, the strains stopped growing. The maximum biomass was closely related to the biomass yield per unit of lactate produced ($Y_{X/P}$) and the MIC (C) of lactate for different homofermentative *Lactobacillus*. Based on the experimental data obtained using different homofermentative *Lactobacillus*, a prediction equation was established as follows: $X_{max} - X_0 = (0.59 \pm 0.02) \cdot Y_{X/P} \cdot C$.

[**Key words:** Homofermentative *Lactobacillus*; Lactate; Acid anions; Maximum biomass; Prediction equation]

Host health is affected by the trophic, protective, and metabolic functions of gut microbiota (1). In humans, *Lactobacillus* species form a pioneer community that prepares the gastrointestinal tract for microbial succession, which continues until microbial maturity is reached (2). In recent decades, *Lactobacillus* has been extensively investigated for prevention and treatment of various digestive diseases (3) and mucosal immune system (4). The list of microorganisms allowed to be used in foods published by the Chinese Ministry of Health in 2010 features 14 species of *Lactobacillus*, of which 12 are homofermentative.

Homofermentative *Lactobacillus* is responsible for the accumulation of high concentrations of extracellular lactic acid and a corresponding decrease in pH. These conditions are known to inhibit spoilage microorganisms and give fermented food favorable sensory properties. Homofermentative species of *Lactobacillus* are commonly used to produce fermented commodities such as dairy products (5), sausages (6), vegetables, and fruits (7). Therefore, it is important to prepare homofermentative-*Lactobacillus* products at high concentrations and with high levels of activity. However, the growth of *Lactobacillus* is strongly inhibited under culture conditions. Lactic acids are the major metabolites of homofermentative *Lactobacillus*. According to Henderson's equation, lactic acid is mainly present in the fermentation broth in the form of undissociated molecule under low pH conditions, which diffuses across the cell membrane passively due to its high solubility in the phospholipid portion of the plasma membrane (8–10). This leads to the dissipation of membrane potential and the acidification of the cytosol, or intracellular anion accumulation (11). However, there was little un-dissociated lactic acid in the fermentation broth cultured at a controlled pH of 7.0, which could have been the most simple and widely applied cultivation. Under these conditions, therefore, the inhibitors were not clear.

In the last few years, the growth of single lactic acid bacteria (LAB) has received considerable attention from researchers, and research groups have modeled the growth kinetics of many strains of LAB (12–15). However, the maximum biomass concentration of *Lactobacillus* was found to differ between strains (16,17) and predicted by different equation. There seems not to be any correlation between different strains of the genus homofermentative *Lactobacillus*.

In the current study, the relationship between maximum biomass and the accumulation of metabolites without substrate inhibition at pH 7.0 was investigated. In addition, the correlation between different strains was analyzed. And an equation for predicting the maximum biomass of homofermentative *Lactobacillus* in fed-batch cultures at pH 7.0 was established.

MATERIALS AND METHODS

Microorganism and inocula *Lactobacillus (L.) plantarum* CCFM 8610, *Lactobacillus plantarum* CCFM 8661, *Lactobacillus acidophilus* CCFM 6, *L. acidophilus* CCFM 137, *Lactobacillus rhamnosus* CCFM 1107, *L. rhamnosus* CCFM 319, *Lactobacillus casei* CCFM 7, *L. casei* CCFM 9, *L. casei* CCFM 30, *Lactobacillus helveticus* CCFM 310, *L. helveticus* CCFM 671, *Lactobacillus bulgaricus* CCFM 4, and *L. bulgaricus* CCFM 29 were obtained from the Culture Collections of Food Microbiology at Jiangnan University (Wuxi, China). *L. plantarum* ATCC 14917 was generously provided by Professor Heping Zhang (Inner Mongolia Agricultural University, China). All of the

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strains are probiotics and are allowed to be used in food. The strains were stored at −80 °C. The colonies isolated from de Man, Rogosa, and Sharpe (MRS) agar were pre-cultured twice in MRS broth, at the respective optimum growth temperatures, for 18 h. *L. acidophilus*, *L. rhamnosus* and *L. casei* were cultured at 37 °C, while *L. plantarum*, *L. helveticus*, and *L. bulgaricus* were cultured at 35 °C, 40 °C and 44 °C, respectively. The bacteria were collected by centrifugation at 5000 *g* at 4 °C for 5 min, washed twice with sterile distilled water, and re-suspended in the same solution. The bacterial suspensions were used as inocula for the fermentation.

Fed-batch cultures at a controlled pH of 7.0 The pH-controlled cultures were grown in 3 L bioreactors (New Brunswick BioFlo 115, Eppendorf, Hamburg, Germany) with 2 L of MRS medium at the respective optimum growth temperatures and an agitation speed of 200 rpm. The filter-sterilized nitrogen was sparged into the culture to keep the tank pressure at approximately 0.03 MPa. The pH was maintained at 7.0 by the automatic addition of a 10 M NaOH solution. The fermenter was inoculated (5%, v/v) and samples were collected every hour, with 5 mL used to monitor the OD_{620} and the remainder centrifuged at 5000 *g* for 5 min. The glucose concentration was measured immediately and the remaining supernatant was frozen at −20 °C until analysis. The bioreactor was initially run in batch mode until a limiting substrate concentration of 5 g L^{-1} was reached. If no feeding medium was added, the residual glucose was almost consumed after 1 h. Thus, the feeding strategy was designed in such a way that the intermittent additions (50–100 mL) were repeated whenever the glucose concentration decreased to a level of 5–10 g L^{-1}, to avoid nutrient limitation. The feeding medium contained 400 g L^{-1} glucose, 100 g L^{-1} beef extract, 100 g L^{-1} peptone, and 50 g L^{-1} yeast extract. Once the bacteria stopped growing, the lactate concentration, osmotic pressure, glucose concentration, and amino nitrogen concentration were analyzed to determine the main factors inhibiting growth.

All of the above cultures were carried out in triplicate. The results presented below are representative of the three fermentations.

Inhibition of homofermentative *Lactobacillus* by acid anions at pH 7.0 Fourteen strains were used to study the influence of acid anions on homofermentative *Lactobacillus* at pH 7.0. The minimum inhibitory concentrations (MICs) of sodium lactate, sodium acetate, and sodium chloride were examined for each strain under neutral conditions. Three kinds of salt were added to the previously prepared MRS medium at 0.1 interval concentrations from 0.1 to 1.5 mmol mL^{-1}. The media were sterilized at 115 °C for 20 min, after which the pH was adjusted to 7.0 by the addition of 2 M sterilized NaOH or HCl in a sterile environment. The osmotic pressure was determined using 1 mL of each sample. The strains were inoculated separately into the media described above and the initial value of the OD_{620} was controlled to approximately 0.05. Next, all of the samples were cultured at the respective optimum growth temperatures for 24 h. Strains with unchanging OD values were considered to be completely inhibited. The osmotic pressure of each medium and the MIC of each strain were measured for three biological replicates to assess reproducibility.

Analytical procedure Cell concentration was monitored by performing plate-culture counts and measuring the optical density of the mediums at 620 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). A correlation between viable-bacteria count and the OD_{620} was established. The samples subjected to optical-density measurement were diluted to ensure that the measured values were between 0.2 and 0.8. Next, the OD_{620} of each sample was calculated as the product of the measured value and the dilution multiple.

The biomass of each strain was quantified by measuring the optical density of fermentation broth at 620 nm. Strain growth was expressed as the viable bacteria count (cfu mL^{-1}), calculated using an equation derived from a calibration curve built from the respective LAB.

A pH meter (FE20—Five Easy, Mettler-Toledo, Zurich, Switzerland) was used to directly determine the pH of each culture. The concentration of amino nitrogen in each culture was detected using formol titration (18). Osmotic pressure was measured using a freezing-point osmometer (LOSER-OM806M, Löser Messtechnik, Berlin, Germany). Glucose concentration was measured using a glucose oxidase Perid test kit (Rsbio, Shanghai, China), according to the manufacturer's instructions. The lactate concentration in each culture was analyzed using high-performance liquid chromatography, as described by Zeng et al. (19).

Statistical methods The experimental results were expressed as means ± standard errors of the means. The linear-regression analysis was carried out using Origin 8.6 software (OriginLab, Northampton, MA, USA). Paired t tests were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) to verify the significant differences. The results were considered significant when $p < 0.05$. The Pearson correlation test was conducted to determine the correlation between the variables using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Growth of strains in fed-batch cultures at a controlled pH of 7.0 The cell growth and pH values of *L. plantarum* ATCC 14917, *L. plantarum* CCFM 8610, *L. plantarum* CCFM 8661, *L. casei* CCFM 7, *L. bulgaricus* CCFM 4, *L. helveticus* CCFM 671, *L. rhamnosus* CCFM 1107, and *L. acidophilus* CCFM 137 in pH 7.0-controlled and fed-batch culture are shown in Fig. 1. Although there were no low pH or un-dissociated lactic acids, the strains reached the point of complete inhibition. At this point, the glucose and amino nitrogen of all the cultures had not been completely consumed (Table 1). Lactate, the main metabolite accumulated, causing the high levels of osmotic pressure in the fermentation broth (Table 1).

Inhibition of homofermentative *Lactobacillus* by acid anions at pH 7.0 It is clear from Table 2 that lactate and acetate had the same MIC values as chloride at pH 7.0 for *L. plantarum*, *L. casei*, *L. helveticus*, *L. bulgaricus* and *L. acidophilus*. *L. helveticus* and *L. acidophilus* were resistance to lower concentrations of acid anions than the others. There was no significant difference between different strains of the same genus. However, different concentrations of acid anions were required to completely inhibit *L. rhamnosus*. The MICs of sodium lactate, sodium acetate, and sodium chloride required to inhibit *L. rhamnosus* increased successively.

Analysis of the difference between the lactate accumulation in the culture and the MIC of lactate Significant differences between the lactate concentration at the point of complete inhibition in the pH 7.0-controlled cultures and the MIC of lactate for the respective strains were verified using a paired t-test, to analyze the main inhibitor. The differences between the osmotic pressure of the fermentation broth at the point of complete inhibition and that of the medium added to the MIC of lactate (Table 3) were tested in a similar way. As shown in Table 4, all *p* values were greater than 0.05.

Development of a prediction equation for maximum biomass The growth of homofermentative *Lactobacillus* is dependent on homolactic fermentation, which is capable of converting 1 mol of glucose to 2 mol of lactic acid, releasing 2 mol of adenosine triphosphate. It is assumed that the biomass concentration relates to the amount of lactic acid produced and the amount of nutrient consumed (20). Growth and production are coupled according to the Leudeking–Piret kinetics equation (21):

$$\frac{dP}{dt} = A \cdot \frac{dX}{dt} + B \cdot X \tag{1}$$

where the constants A and B are the coefficients for growth- and non-growth-associated production respectively. If product formation is growth associated, $A \neq 0$ and $B0 = 0$. During the logarithmic (log) phase of homofermentative *Lactobacillus*, a significant relationship was found between the lactate accumulation and cell growth (all strains: $p < 0.01$, $R > 0.99$). Thus, Eq. 1 can be written as

$$\frac{dP}{dt} = Y_{P/X} \cdot \frac{dX}{dt} \tag{2}$$

It can also be written as

$$\frac{dX}{dt} = Y_{X/P} \cdot \frac{dP}{dt} \tag{3}$$

where $Y_{X/P}$ was estimated from the slope obtained by plotting the cell density against the lactate concentration in the log phase of the batch culture, using linear regression analysis. It was assumed that the strain was in log growth until the point of complete inhibition. Thus Eq. 3 can be modified and solved using an integrated form given as: