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Modeling the cucumber fermentation: growth of *Lactobacillus plantarum*

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SUMMARY

Specific growth rate models of product-inhibited cell growth exist but are rarely applied to fermentations beyond ethanol and large-scale antibiotic production. The present paper summarizes experimental data and the development of a model for growth of the commercially important bacterium, *Lactobacillus plantarum*, in cucumber juice. The model provides an excellent correlation of data for the influence on bacterial growth rate of NaCl, protons (H^+), and the neutral, inhibitory forms of acetic acid and the fermentation product, lactic acid. The effects of each of the variables are first modeled separately using established functional forms and then combined in the final model formulation.

INTRODUCTION

Product-inhibited fermentations are commonly found in commercial production of antibiotics, wines, alcohol for motor fuels, and acidic products such as cheeses and pickles. While simple models for inhibition by a single environmental solute (e.g., antibiotic, pH, acid product, ethanol, etc.) have been proposed (Table 1), very few studies have attempted to model a product-inhibited fermentation involving multiple inhibition variables. Kinetic models of food processes are rare in the literature, probably as a result of the complexity of such systems. Such models can be used for prediction of microbial safety or shelf life of products, detection of critical points of the production and distribution processes, optimization of production and distribution chains [11], and control of fermentation processes.

In the present study, growth rates of *Lactobacillus plantarum* in cucumber juice were measured and the influences of four environmental variables (NaCl, pH, and the undissociated forms of lactic and acetic acids) were modeled.

TABLE 1

Model equations for cell growth under product inhibited conditions

Kinetic model, f (C)	Form	Reference
$\mu = \mu_{max}\left[1 - \frac{[C]}{[C]_{max}}\right]$	Linear	[4]
$\mu = \mu_{max}\left[1 - \frac{[C]}{[C]_{max}}\right]^{\alpha}$	Non-linear	[7]
$\mu = \mu_{max}\left[1 - \left(\frac{[C]}{[C]_{max}}\right)^{\beta}\right]$	Non-linear	[8]
$\mu = \mu_{max}\exp(-K_i[C])$	Exponential	[2]
$\mu = \mu_{max}\left(\frac{K_i}{K_i + [C]}\right)$	Hyperbolic	[1]
$\mu = \mu_{max}\left(\frac{K_{i1}}{K_{i1} + [C]} - K_{i2}[C]\right)$	Non-linear	[6]

MATERIALS AND METHODS

Culture

Lactobacillus plantarum MOP-3 isolated previously from commercial cucumber fermentations [3] provided in this laboratory was stored in MRS broth containing 16% glycerol at −70 °C. Isolated colonies from MRS agar streak plates were picked and grown twice at 30 °C in cucumber juice for

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TABLE 2

Combinations of NaCl and acetic and lactic acids used to test growth prediction Eqn 2

Treatment	NaCl, %	$[Ac_t]$, mM	$[La_t]$, mM	pH	[HAc], mM	[HLa], mM
A	3	40	10	3.83	35.7	5.1
B	5	10	10	4.14	8.0	3.4
C	3	40	10	3.82	35.8	5.2
D	5	10	10	4.08	8.2	3.8
E	3	40	28	3.53	37.7	19.1
F	5	10	28	3.57	9.4	18.5
G	3	40	28	3.52	37.8	19.2
H	5	10	28	3.57	9.4	18.5
I	0	5	0	5.14	1.4	0
J	0	10	0	4.85	4.4	0
K	0	0	10	4.59	0	1.6
L	0	0	30	3.86	0	15.0
M	3	0	0	5.54	0	0
N	5	0	0	5.49	0	0
P	8	0	0	5.41	0	0
R	9	0	0	5.38	0	0
S	4	5	0	4.90	2.1	0
T	4	40	0	4.02	33.7	0
U	6	40	0	3.89	35.1	0

See Fig. 4 for the relationship between observed and predicted specific growth rate values.

12–15 h. Inocula were diluted to optical density ($OD_{630\ nm}$) of 0.4–0.5 and 1.0% (v/v) was added to each growth medium studied to give an initial concentration of around 10^6 cells ml^{-1}.

Growth media

Cucumber juice was prepared, centrifuged, diluted, and filter-sterilized (0.22 μm), following procedures given in detail by Passos [9]. Hydrochloric acid, lactic acid, acetic acid, and NaCl were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI).

Fermentors

Water-jacketed, 200-ml jars (Wheaton, Millville, NJ) were used for uncontrolled pH, batch growth systems with continuous nitrogen flow over the headspace (2.5 ml min^{-1}) to ensure anaerobic conditions. Samples (3 ml) were removed aseptically by syringe at 1–6 h intervals, depending on cell growth rate. OD and pH measurements were made, and the samples were then frozen until later HPLC analysis.

Analytical methods

Standard methods for determination of cell concentration (OD), NaCl ($AgNo_3$/fluorescein titration) and organic acids (HPLC) are described elsewhere [9].

MODEL DEVELOPMENT

The kinetics of cell growth can be described by modeling the cell batch specific growth rate μ, defined as:

$$\mu \equiv \frac{1}{X}\frac{dX}{dt} \tag{1}$$

where X = cell concentration. Assuming that the influences of the four inhibitor variables are independent, we may write

$$\mu = \mu_0 f_1([H^+])\, f_2([HLa])\, f_3([HAc])\, f_4([NaCl]) \tag{2}$$

where f_1, f_2, f_3, and f_4 refer to the presumably independent influences of protons, neutral forms of lactic and acetic acids ([HLa] and [HAc], respectively), and [NaCl]. This independence is tested following the establishment of suitable functions for f_1 through f_4.

To define the mathematical relationship between specific growth rate and component concentrations, several models were fitted to the data by nonlinear regression using software SAS (SAS Institute, Cary, NC), Procedure NLIN, which implements the iterative modified Gauss–Newton method (SAS, 1988). The goodness-of-fit criteria used to select the best model from Table 1 was the least square values, except when no Table 1 model was applicable (e.g., for influence of salt and of acetic acid). The parameters $[C]_{max}$, μ_{max}, and coefficients α and β were estimated by this model-fitting procedure. Table 1 presents the functions tested.

The four subsections that follow discuss the individual effects on cell growth rate of hydrogen ion, nonionized lactic and acetic acids, and NaCl. The final subsection treats the combined effects of these variables.

Proton (H^+)

Hydrochloric acid (HCl) was used to vary the initial medium pH for measurements of initial cell growth rate versus pH in the absence of other inhibitors. The data obtained (Fig. 1) are fit best by an inhibition function proposed by Levenspiel [7] (Table 1). The solid line in Fig. 1 is calculated using the function

$$\mu = \mu_0 f_1([H^+]) \tag{3}$$

where $\mu_0 = 0.35\ h^{-1}$ and

$$f_1([H^+]) = \left(1 - \frac{[H^+]}{[H^+]_{max}}\right)^{\alpha_1} \tag{4}$$

where $[H^+]$ is expressed in mM, $[H^+]_{max} = 0.43$ mM, and $\alpha_1 = 2.6$. The most acidic pH in Fig. 1 (3.37) corresponds to the value at which batch growth ceased, during the tests conducted.

Nonionized lactic acid (HLa)

Kuhn [6] recently established that effects of acid-product-inhibited *Escherichia coli* could be decomposed into the separate influences of pH and the nonionized forms of the products (acetate and formate). Yabannavar and Wang [10] showed that for *L. delbrueckii* the growth-inhibiting effect of the ionized form of lactic acid is extremely small when

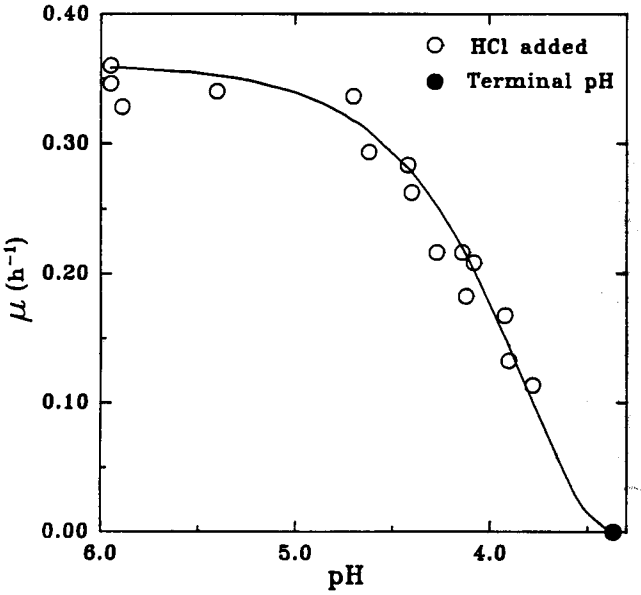

Fig. 1. Effect of pH on the specific growth rate of *L. plantarum*. The solid line represents values predicted using Eqn 3. ([9], with permission.)

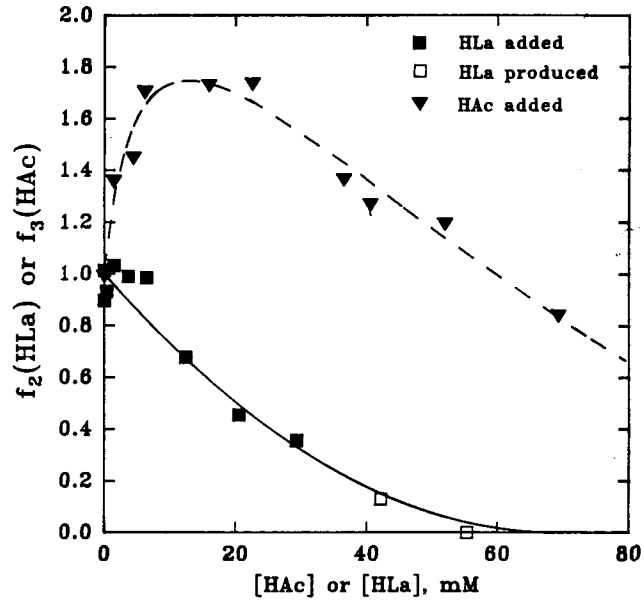

Fig. 2. Effect of undissociated lactic and undissociated acetic acid concentrations on the specific growth rate of *L. plantarum*. The solid and dashed lines represent values predicted using Eqns 7 and 10. (Modified from [9], with permission.)

compared with that of nonionized lactic acid and proposed a model relating specific growth rate to concentrations of hydrogen ion and the nonionized form of lactic acid. It is well known that only the uncharged forms of organic acids can penetrate the cell membrane [5]; thus, [HLa] and [HAc] were of primary interest in this study. To model the influence of [HLa], we used the acid ionization equilibrium,

$$[\mathrm{HLa}] \rightleftarrows [\mathrm{H^+}] + [\mathrm{La^-}] \tag{5}$$

to calculate [HLa] from measurements of total lactic acid ([HLa] + [La^-]). The corresponding effect of [HLa] on the measured overall specific growth rate was estimated after subtracting out the [H^+] effect. As with [H^+], the simple inhibition model of Levenspiel [7] provided the best fit of all Table 1 models, giving

$$\mu = \mu_0 f_1([\mathrm{H^+}])\, f_2([\mathrm{HLa}]) \tag{6}$$

where [HLa] (mM) is the concentration of nonionized lactic acid,

$$f_2([\mathrm{HLa}]) = \left(1 - \frac{[\mathrm{HLa}]}{[\mathrm{HLa}]_{max}}\right)^{\alpha_2} \tag{7}$$

$[\mathrm{HLa}]_{max}$ = 69 mM, and α_2 = 2.0. The data (squares) and model Eqn 7 (solid line) are shown in Fig. 2. The filled points represent initial growth rates determined from direct lactic acid addition to cucumber juice. Growth was not observed at initial pH values below 3.7; however, in media where *L. plantarum* had already initiated substantial growth, growth continued until the pH decreased as low as 3.37. To obtain a more realistic mathematical representation of the process, specific growth rates measured during the deceleration growth phase were included in the analyses (open circles).

Nonionized acetic acid (HAc)

Acetate addition first increased, then decreased the specific growth rate. Thus, acetate functions as stimulant or inhibitor of growth, depending upon its concentration. None of the models in Table 1 account for such dual functional behavior, but an empirical function (Eqn 8) serves this purpose:

$$f([\mathrm{C}]) = \underbrace{\left(1 + \frac{\beta\,[\mathrm{C}]}{K_m^{\mathrm{C}} + [\mathrm{C}]}\right)}_{\text{Stimulation}} \underbrace{\left(1 - \frac{[\mathrm{C}]}{[\mathrm{C}]_{max}}\right)}_{\text{Inhibition}} \tag{8}$$

where [C] is the stimulatory-inhibitory component concentration. As seen in Fig. 2, Eqn 8 provided a good fit of the data, giving

$$\mu = \mu_{max} f_1([\mathrm{H^+}])\, f_3([\mathrm{HAc}]) \tag{9}$$

where

$$f_3([\mathrm{HAc}]) = \left(1 + \frac{\beta_1\,[\mathrm{HAc}]}{K_m^{\mathrm{HAc}} + [\mathrm{HAc}]}\right)\left(1 - \frac{[\mathrm{HAc}]}{[\mathrm{HAc}]_{max}}\right)^{\alpha_3} \tag{10}$$

The fitted parameters were β_1 = 1.5, K_m^{HAc} = 5.8 mM, $[\mathrm{HAc}]_{max}$ = 150 mM, and α_3 = 1.7.

NaCl

As with acetic acid, low NaCl addition enhanced growth (Fig. 3) and higher concentrations led to inhibition with no

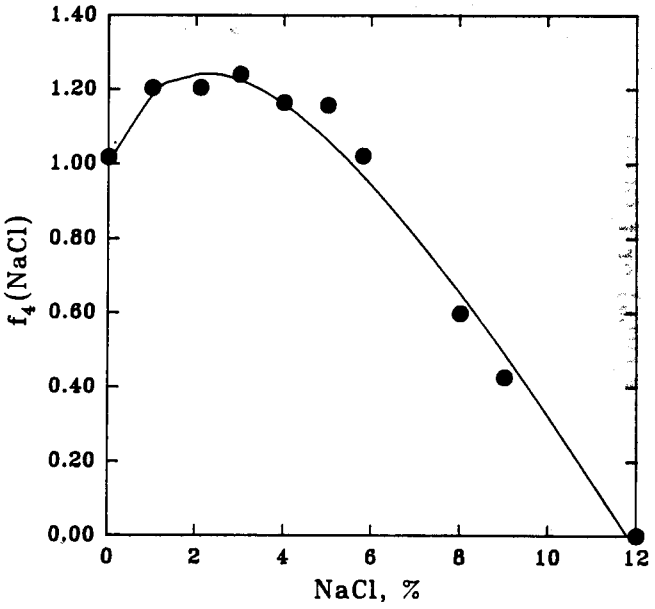

Fig. 3. Effect of NaCl concentration on the specific growth rate of *L. plantarum*. The solid curve represents values predicted using Eqn 12. (Modified from [9], with permission.)

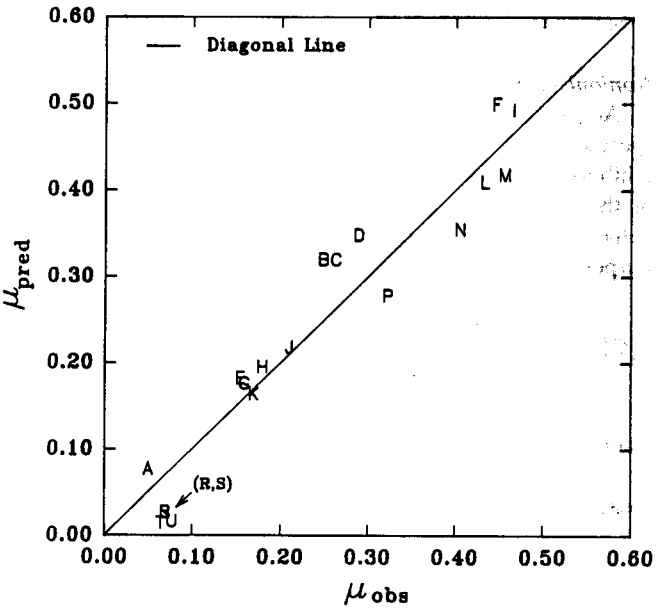

Fig. 4. The relationship of specific growth rate of *L. plantarum* predicted using Eqn 2 and observed specific growth rates in 19 independent fermentations using different combinations of initial concentrations of lactic acid, acetic acid, and NaCl (Table 2). ([9], with permission.)

growth achieved at 12% NaCl. The function f_4 is of the same form as Eqn 8:

$$\mu = \mu_{max} f_4([NaCl]) \tag{11}$$

where [NaCl] is the weight percent of NaCl,

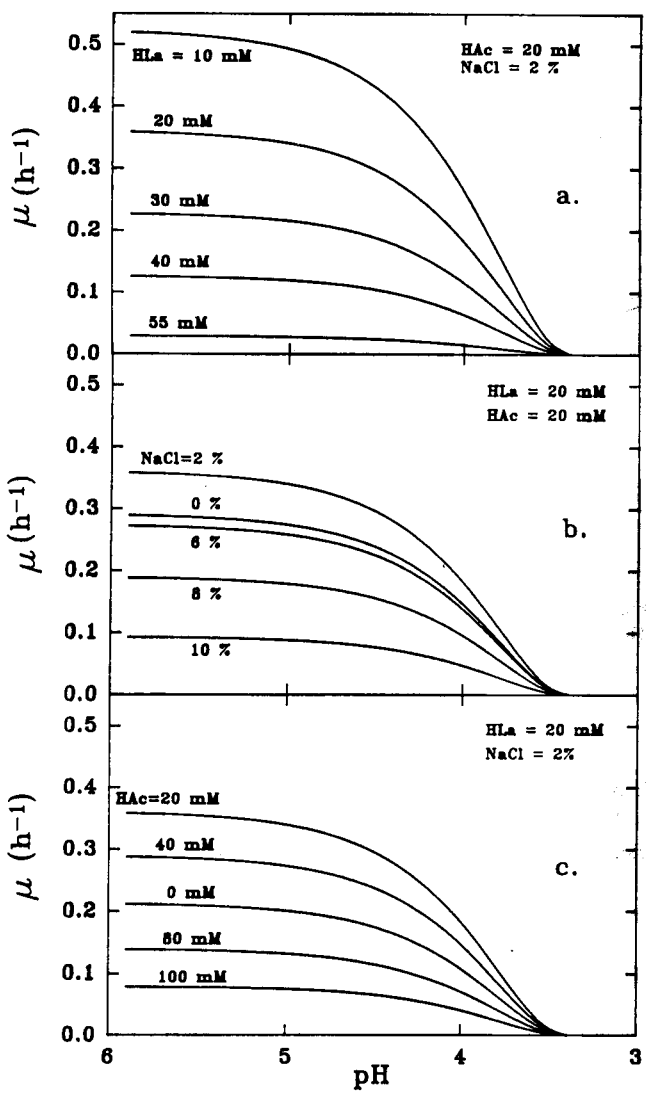

Fig. 5. Predicted effect of pH on the specific growth rate of *L. plantarum*. (a) Variable concentrations of undissociated lactic acid, 20 mM undissociated acetic acid, 2% NaCl. (b) Variable NaCl concentrations, 20 mM undissociated lactic and acetic acids. (c) Variable concentrations of undissociated acetic acid, 20 mM undissociated lactic acid, 2% NaCl. ([9], with permission.)

$$f_4([NaCl]) = \left(1 + \frac{\beta_2[NaCl]}{K_m^{NaCl} + [NaCl]}\right)\left(1 - \frac{[NaCl]}{[NaCl]_{max}}\right) \tag{12}$$

and $\beta_2 = 1.6$, $K_m^{NaCl} = 4.47\%$, and $[NaCl]_{max} = 11.8\%$. The solid line in Fig. 3 shows good agreement with the data.

Test of independence of [H$^+$], [HLa], [HAc], and [NaCl] effects

Effects of the different inhibitory components on cell metabolism and growth are necessarily coupled to some extent; however, the simplest circumstance to model is that of independent influences, as assumed in the present paper.

The functions f_1–f_4 of Eqn 2 were determined from

experimental cell growth measurements in which only a single inhibiting component was present in the growth medium. The assumed independence of the inhibiting effects was tested by measuring growth rates in the presence of combinations of the four inhibitory components and correlating the data with Eqn 2 without adjusting the four functions from their individually determined values. The results are shown in Fig. 4. The goodness-of-fit of the observed values against the predicted values was subjected to a chi-square test; the fit was significant at the 0.005 level. The excellent agreement between the predicted and measured growth rates over a broad range of conditions justifies the assumption of independence.

Growth rate prediction

With the apparent independence of the four variables ($[H^+]$, [HLa], [HAc], and [NaCl]) established, the specific growth rate can now be predicted as a function of all variables. Sample calculations are presented in Fig. 5 for various ranges of pH, NaCl, and acetic acid and lactic acid concentrations.

The influence of fermentation products on specific growth rate and the coupling of biological kinetics to mass transfer of solutes to and from the cucumber will be considered in subsequent papers.

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NOMENCLATURE

[C]	inhibitory component concentration, mM
$[C]_{max}$	concentration of the inhibitory component where the specific growth rate is zero, mM, determined by model fitting
$[H^+]$	hydrogen ion concentration, mM
[HLa]	undissociated lactic acid concentration, mM
$[La^-]$	dissociated lactic acid concentration, mM
$[La_t]$	total lactic acid ([HLa] + $[La^-]$) concentration, mM
[HAc]	undissociated acetic acid concentration, mM
$[Ac^-]$	dissociated acetic acid concentration, mM
$[Ac_t]$	total acetic acid ([HAc] + $[Ac^-]$) concentration, mM
[NaCl]	sodium chloride concentration, %, w/v
μ	specific growth rate, h^{-1}
μ_{max}	maximum specific growth rate, h^{-1}
μ_0	specific growth rate, h^{-1}, at 0 concentration of additive
K_{ij}	inhibition coefficients
α, β, K_m	coefficients determined by model fitting