



# Modeling growth of *Saccharomyces rosei* in cucumber fermentation†

Frederico V. Passos<sup>1</sup>, Henry P. Fleming<sup>2\*</sup>, Richard M. Felder<sup>3</sup> and David F. Ollis<sup>3</sup>

*Objectives of this study were to assess the effects of key variables involved in cucumber fermentation on growth of the yeast, Saccharomyces rosei, and to develop a mathematical description of those effects. The growth medium for the studies was cucumber juice. Effects of concentrations of lactic, acetic, and hydrochloric acids and sodium chloride on growth at 30°C were determined in batch culture. Effect of substrate concentration on the specific growth rate was also defined. The specific growth rate decreased from 0.355 h<sup>-1</sup> at pH 6.0 to 0.189 h<sup>-1</sup> at pH 3.2. The undissociated form of lactic acid was more inhibitory than that of acetic acid. A predictive equation for specific growth rate was developed for predicting growth of S. rosei in batch culture. The molar yield of ethanol was 1.75 (±0.07) mM ethanol per mM hexose. Malate was not utilized, and glycerol was produced. The apparent biomass yield under anaerobic condition was 12.2 (±1.3) g cells/mol hexose. Aerobically, the biomass yield was 30.7 g cells/mol hexose. Similar specific growth rates were observed anaerobically (0.358 h<sup>-1</sup>) and aerobically (0.352 h<sup>-1</sup>). The predictive model for growth of S. rosei in cucumber juice should prove useful in modeling the mixed culture (yeast and lactic acid bacteria) fermentation of brined, whole cucumbers.*

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<sup>1</sup>Department of Food Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil 36570.

<sup>2</sup>Food Fermentation Laboratory, U.S. Department of Agriculture, Agricultural Research Service, and North Carolina Agricultural Experiment Station, Raleigh, North Carolina U.S.A. 27695-7624

<sup>3</sup>Department of Chemical Engineering, N. C. State University, Raleigh, NC, U.S.A. 27695-7905.

## Introduction

In cucumber fermentation, preservation results from the conversion of the fruit sugars into lactic acid and other compounds and by a lowering of the pH. Cucumber nutrients available for the fermentation must diffuse through the tissue and skin of the fruit into the surrounding brine. Also, sodium chloride and acetic acid added in the begin-

ning of the process diffuse into the fruit. The concentration of these components will exert selective effects on growth of the natural microflora and/or on the starter culture during fermentation. In addition, the mass transfer rate of solutes affects the growth rate of micro-organisms.

Fermentations by homolactic acid bacteria alone result in concentrations of lactic acid which are too high for direct consumption of the fermented product. A minimum of 0.6% (66.7 mM) lactic acid has been recommended to insure preservation (Etchells and Hontz 1972). Excessive acidity levels, however, can adversely affect the texture (Thompson et al. 1979) and flavor (Daeschel et al. 1988) of the fermented cucumbers. In addition, sugar may remain after primary fermentation by lactic

\*Corresponding author.

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acid bacteria, resulting in subsequent fermentation and bloater damage by acid-tolerant, fermentative yeast.

The use of buffer components or neutralization of the acid produced to allow complete conversion of sugar to end products by lactic acid bacteria has been recommended (Etchells et al., 1973). The addition of a fermentative yeast, *Saccharomyces rosei*, has been suggested as a means of partially utilizing fermentable sugars to avoid excessive acid production by lactic acid bacteria (Daeschel et al. 1988). Although yeast and other gas-forming bacteria can cause bloater damage to the cucumber, brines can be purged of the CO<sub>2</sub> produced using air or N<sub>2</sub> to avoid this problem (Fleming et al. 1975).

The objective of this research was to develop a mathematical model to predict the growth of *S. rosei*. Effects of lactic acid, acetic acid, NaCl, and hydrogen ions on the specific growth rate were considered. Growth equations developed for *S. rosei*, when combined with similar equations defined for *Lactobacillus plantarum* and equations for diffusion of soluble components in/out of the fruit, will provide a kinetic model for mixed culture fermentations of whole cucumbers.

## Materials and Methods

### *Culture and growth medium*

The yeast used in the study was *S. rosei*, isolated from fermenting cucumber (Daeschel et al. 1988) and stored in YM broth containing 16% glycerol at -70°C. Isolated colonies were picked from YM agar streak plates of the frozen culture and grown twice in cucumber juice for 12–15 h at 30°C. The inoculum growth medium was supplemented with acetic acid (40 mM), lactic acid (40 mM), or NaCl (4%) as needed to be consistent with the growth medium. The inoculum culture was diluted to an optical density (OD<sub>630 nm</sub>) of 0.4–0.5, and 1.0% (by volume) in the growth medium to give initial cell levels approximating 5×10<sup>5</sup>/ml.

Cucumber juice for growth studies was prepared as previously described (Passos et al. 1993). The chemicals used in the study

were hydrochloric acid, DL-lactic acid, acetic acid (Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.) and sodium chloride (Fisher Scientific, Pittsburgh, PA, U.S.A.).

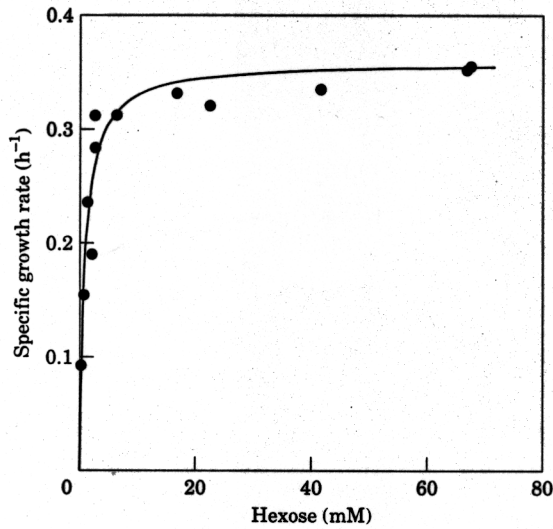
Cucumber juice at different dilutions was used to test the effect of substrate concentration on the specific growth rate of *S. rosei*. Media containing 0.1 to 50.0% undiluted cucumber juice were prepared.

### *Fermentors*

Water-jacketed jars from Wheaton (Millville, NJ, U.S.A.), with 200 ml working volume, were used as uncontrolled pH batch growth systems. The growth medium was agitated by a magnetic stirrer and maintained at 30°C. Compressed N<sub>2</sub> was humidified, sterilized (0.22 µm Millex-FG<sub>50</sub> filter, Millipore Corp., Bedford, MA, U.S.A.), and released into the head space of the fermentor at a rate of 2.5 l/h to assure anaerobic conditions in all the experiments. During batch growth, 3-ml samples were removed aseptically by syringe from the 200 ml initial broth volume at intervals of 1–2 h (depending on the fermentation rate) until growth ceased, used for optical density and pH measurement, and then frozen for future HPLC analysis.

### *Analytical methods*

Cell growth was followed by measurement of the OD of the medium in a 1.5 ml glass cuvette using a Novaspec II spectrophotometer (Pharmacia LKB, Piscataway, NJ, USA). The linear range extended to OD readings of 0.30. During growth, if the OD was higher than 0.25, the sample was diluted to within a range of 0.10 to 0.25 using distilled water. Standard curves were used to relate OD, dry weight (g/l) and cell number (CFU/ml). For dry weight determination a 500 ml cell suspension (around 0.6 OD) was washed two times with an equal volume of sterile water, concentrated 25× by centrifugation, and 4 samples of 3 ml each were then dried to constant weight in a vacuum oven at 80°C. Viable cells were enumerated in YM agar (Difco Labs, Detroit, MI, U.S.A.), using the same cell suspension used for dry weight. One unit of OD was equivalent to 0.39 g



**Figure 1.** Effect of substrate concentration on the specific growth rate of *S. rosei*.

cells/l and  $1.5 \times 10^7$  CFU/ml, for dry weight and cell number, respectively. All the chemicals used were described in Passos et al. (1993). Initial specific growth rates were calculated from linear regression analysis of the exponential portion of the initial growth curves.

### Model development

To develop a mathematical representation of the specific growth rate of *S. rosei* as a function of the dynamic chemical variables during cucumber fermentation (pH, lactic acid, acetic acid, and NaCl concentrations), the effect of each variable was studied individually. The kinetics of cell growth can be described by modeling the cell batch specific growth rate  $\mu$ , defined as:

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad (1)$$

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

$$\mu = \mu_0 f_1([S]) f_2([H^+]) f_3([HLA]) f_4([HAc]) f_5([NaCl]) \quad (2)$$

where  $f_1$ ,  $f_2$ ,  $f_3$ ,  $f_4$ , and  $f_5$  refer to the presum-

ably independent influences of substrate limitation ( $[S]$ ), protons ( $[H^+]$ ), undissociated 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  $f_5$ .

To define the mathematical relationship between specific growth rate and component concentrations, models were fitted to the data using non-linear or linear regression and goodness-of-fit criteria. Resultant data indicated the best model for establishing relevant coefficients. Analysis was performed with SAS software using the iterative, modified Gauss-Newton method for non-linear analyses (proc NLIN) and the principle of least squares for linear analyses (proc REG) (SAS 1988). Each model was selected using the value of the sum of squares as criteria.

## Results

### Substrate limitation ( $S$ )

The Monod model (equations 3 and 4) was applied to describe the hexose concentration effect on the specific growth rate of *S. rosei*. The values of  $K_m$  and  $\mu_{max}$  were determined as 0.86 mM and  $0.359 \text{ h}^{-1}$ , respectively, using a Lineweaver-Burk plot (Bailey and Ollis 1986). Figure 1 shows the experimental data (symbols) and fitted model (line) for the growth rate ( $\mu$ ), where

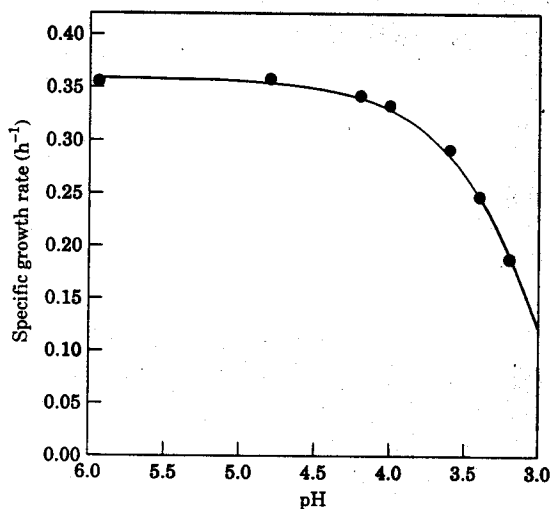
$$\mu = \mu_{max} f_1([S]) \quad (3)$$

and

$$f_1([S]) = \left( \frac{[S]}{K_m + [S]} \right) \quad (4)$$

### Proton concentration effect ( $[H^+]$ )

Hydrochloric acid (HCl) was used to vary the initial medium pH for measurements of initial cell growth rate vs pH in the absence



**Figure 2.** Effect of pH on the specific growth rate of *S. rosei*.

of other inhibitors. The data obtained (Fig. 2) are fit best by an inhibition function proposed by Levenspiel (1980). The solid line in Fig. 2 was calculated using the function

$$\mu = \mu_{\max} f_2([H^+]) \quad (5)$$

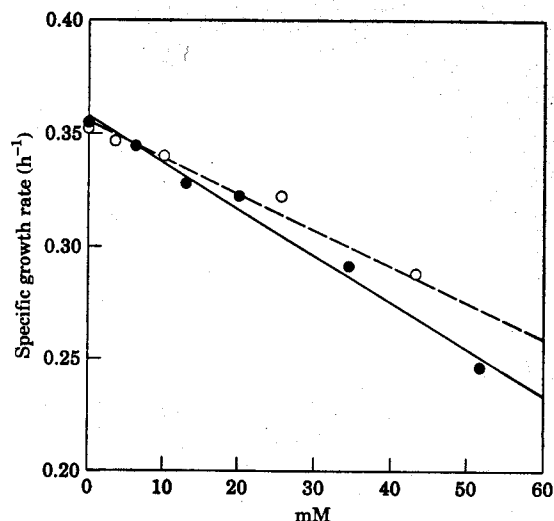
where  $\mu_{\max} = 0.359 \text{ h}^{-1}$  and

$$f_2([H^+]) = \left(1 - \frac{[H^+]}{[H^+]_{\max}}\right)^{\alpha} \quad (6)$$

where  $[H^+]$  is expressed in mM,  $[H^+]_{\max} = 2.50 \text{ mM}$ , and  $\alpha = 2.13$ .

#### Undissociated lactic acid concentration effect ([HLa])

Kuhn (1991) recently established that effects of acid-product-inhibited *Escherichia coli* could be decomposed into the separate influences of pH and the undissociated forms of the products (acetate and formate). A similar approach was used to evaluate the effect of acetic and lactic acids on the growth of *L. plantarum* (Passos et al. 1993). Yabannavar and Wang (1991) showed that for *L. delbrueckii* the growth-inhibiting effect of the ionized form of lactic acid is extremely small when compared with that of non-ionized lactic acid and proposed a model relating specific growth rate to concentrations of



**Figure 3.** Effects of undissociated lactic and undissociated acetic acid concentrations on the specific growth rate of *S. rosei*: [HLa] (●), [HAc] (○).

hydrogen ion and the non-ionized form of lactic acid. It is well known that only the uncharged forms of organic acids can penetrate the cell membrane (Ingram et al. 1956); thus, [HLa] and [HAc] were of primary interest in this study. To model the influence of [HLa], we used the acid ionization equilibrium,

$$[HLa] = [H^+] + [La^-] \quad (7)$$

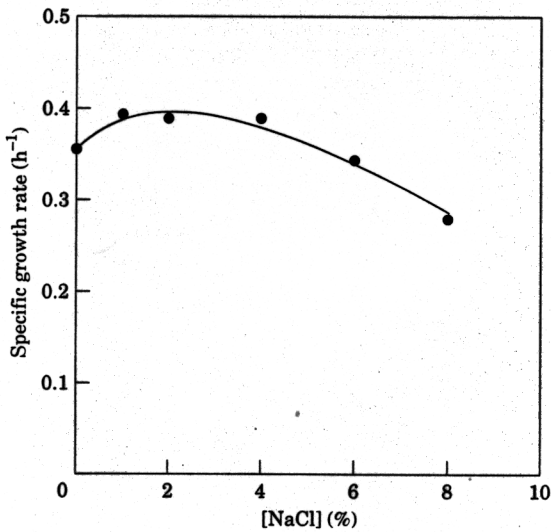
to calculate [HLa] from measurements of total lactic acid ( $[HLa] + [La^-]$ ). The corresponding effect of [HLa] on the measured overall specific growth rate was estimated after subtracting out the  $[H^+]$  effect. A simple linear inhibition model provided the best fit in the range studied, giving

$$\mu = \mu_{\max} f_2([H^+]) f_3([HLa]) \quad (8)$$

where [HLa] (mM) is the concentration of non-ionized lactic acid,

$$f_3([HLa]) = \left(1 - \frac{[HLa]}{[HLa]_{\max}}\right) \quad (9)$$

and  $[HLa]_{\max} = 156 \text{ mM}$ . The experimental data (symbols) and predicted values from Eqn 8 (solid line), are shown in Fig. 3.



**Figure 4.** Effect of NaCl concentration on the specific growth rate of *S. rosei*.

#### Non-ionized acetic acid concentration effect ([HAc])

The same equation used for [HLA] (Eqn 9) was also used to describe the inhibitory effect of [HAc]:

$$\mu = \mu_{\max} f_2 ([H^+]) f_4 ([HAc]) \quad (10)$$

where

$$f_4 ([HAc]) = \left( 1 - \frac{[HAc]}{[HAc]_{\max}} \right) \quad (11)$$

The fitted parameter was  $[HAc]_{\max} = 218$  mM. The experimental data (symbols) and predicted values from Eqn 10 (broken line) are shown in Fig. 3.

#### NaCl concentration effect

NaCl addition first increased, then decreased the specific growth rate of *S. rosei*. Thus, salt functions as stimulant or inhibitor of growth, depending upon its concentration. A similar behavior was observed for *L. plantarum* (Passos et al. 1993) and the same function was used to account for such dual functional behaviour:

$$f ([C]) = \left( 1 + \frac{\beta [C]}{K_M^C + [C]} \right) \left( 1 - \frac{[C]}{[C]_{\max}} \right) \quad (12)$$

where [C] is the stimulatory-inhibitory component concentration. As seen in Fig. 4, Eqn 12 provided a good fit of the data, giving

$$\mu = \mu_{\max} f_5 ([NaCl]) \quad (13)$$

where [NaCl] is the weight percent of NaCl,

$$f_5 ([NaCl]) = \left( 1 + \frac{\beta [NaCl]}{K_{NaCl} + [NaCl]} \right) \left( 1 - \frac{[NaCl]}{[NaCl]_{\max}} \right) \quad (14)$$

and  $\beta = 0.8$ ,  $K_{NaCl} = 4.1\%$ , and  $[NaCl]_{\max} = 16.9\%$ .

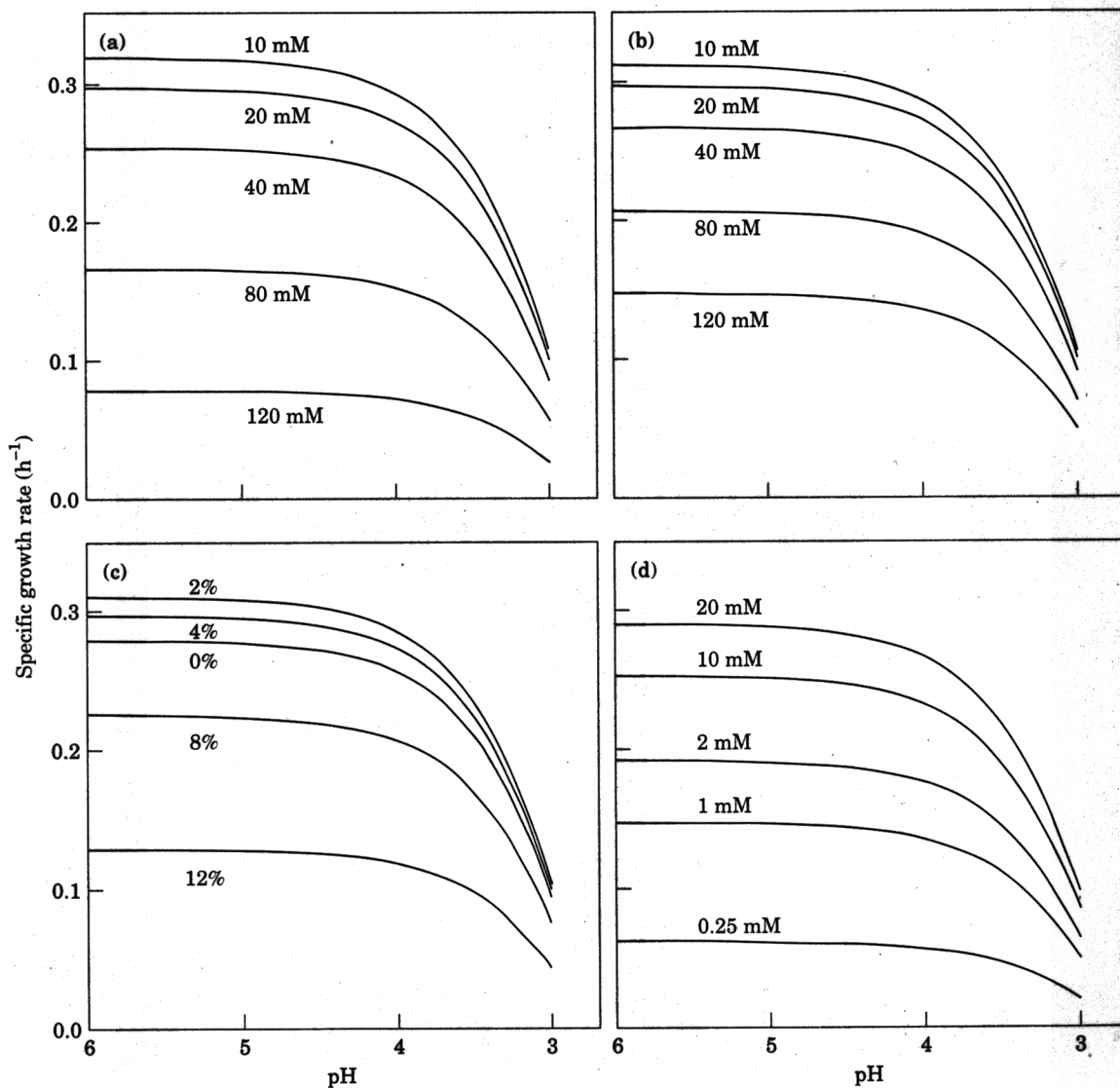
#### Test of independence of [H<sup>+</sup>], [HLA], [HAc], and [NaCl] effects

The functions  $f_2$ – $f_5$  of Eqn 2 were determined from experimental cell growth measurements in which only a single inhibiting component was present in the growth medium. The presumed independence of the inhibiting effects was tested by measuring growth rates in the presence of combinations of the four components and correlating the data with Eqn 2, without adjusting the four factors from their individually determined values. The results are shown in Table 1. The goodness-of-fit of the observed values against the predicted values was subjected to a  $\chi^2$  test; the fit was significant at the 0.005 level. The excellent agreement between the predicted and measured growth rates over the range of conditions tested justifies the assumption of independence.

Model-predicted variations in growth rate over ranges of pH, NaCl, and lactic and acetic acid concentrations are shown in Fig. 5. In Fig. 5a, undissociated acetic acid, NaCl, and hexose concentrations were kept constant, and the effect of pH was demonstrated for different undissociated lactic acid concentrations. In Fig. 5b, undissociated lactic acid, NaCl, and hexose were kept constant, and the effect of pH was demonstrated for different undissociated acetic acid concentrations. In Fig. 5c, undissociated lactic acid, undissociated acetic acid, and hexose concentrations were kept constant, and the effect of

**Table 1.** Combinations of acetic and lactic acids and NaCl used to test growth prediction Eqn 2.

pH	[La <sub>i</sub> ], mM	[Ac <sub>i</sub> ], mM	NaCl, %	$\mu_{\text{obs}}$	$\mu_{\text{pred}}$
4.38	0	18	3	0.362	0.362
4.15	0	35	3	0.259	0.330
3.74	15	18	6	0.227	0.264
3.41	38	18	3	0.203	0.230
3.82	20	10	2	0.335	0.325
3.36	55	25	2	0.241	0.204
3.21	55	25	6	0.145	0.127
5.92	0	0	0	0.359	0.359



**Figure 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, 4% NaCl, 50 mM hexose; (b) variable concentrations of undissociated acetic acid, 20 mM undissociated lactic acid, 4% NaCl, and 50 mM hexose; (c) variable NaCl concentrations, 20 mM undissociated lactic and acetic acids, and 50 mM hexose; (d) variable concentrations of hexose, 20 mM undissociated lactic and acetic acids, and 4% NaCl.