Journal of Food Protection, Vol. 63, No. 2, 2000, Pages 222–230
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The Boundary for Growth of *Zygosaccharomyces bailii* in Acidified Products Described by Models for Time to Growth and Probability of Growth

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MS 99-50: Received 23 February 1999/Accepted 24 September 1999

ABSTRACT

Models to predict days to growth and probability of growth of *Zygosaccharomyces bailii* in high-acid foods were developed, and the equations are presented here. The models were constructed from measurements of growth of *Z. bailii* using automated turbidimetry over a 29-day period at various pH, NaCl, fructose, and acetic acid levels. Statistical analyses were carried out using Statistical Analysis Systems LIFEREG procedures, and the data were fitted to log-logistic models. Model 1 predicts days to growth based on two factors, combined molar concentration of salt plus sugar and undissociated acetic acid. This model allows a growth/no-growth boundary to be visualized. The boundary is comparable with that established by G. Tuynenburg Muys (Process Biochem. 6:25–28, 1971), which still forms the basis of industry assumptions about the stability of acidic foods. Model 2 predicts days to growth based on the four independent factors of salt, sugar, acetic acid, and pH levels and is, therefore, much more useful for product development. Validation data derived from challenge studies in retail products from the U.S. market are presented for Model 2, showing that the model gives reliable, fail-safe predictions and is suitable for use in predicting growth responses of *Z. bailii* in high-acid foods. Model 3 predicts probability of growth of *Z. bailii* in 29 days. This model is most useful for spoilage risk assessment. All three models showed good agreement between predictions and observed values for the underlying data.

A wide range of sauces, ketchups, pickles, mustards, mayonnaises, salad dressings, and similar products are usually considered to be shelf stable. Typical pH values for these products are in the range of 3.0 to 4.0, and they would be considered high-acid foods for the purpose of thermal processing *(14)*. Thermal processes may be applied to these types of foods, but their stability is based primarily on the combined effects of acidity, salt, and sugar. Acetic acid (as vinegar) is the most common acidulent in these products. Manufacturers also employ good sanitation and may use chemical preservatives and other barriers, such as refrigeration, to prevent microbial growth. Traditional formulations of these products are intrinsically resistant to growth of pathogens but are susceptible to spoilage by lactic acid bacteria and yeasts, including *Zygosaccharomyces bailii (7, 9, 13, 15)*. Over many years, the consumer trends in the United States and Europe have been toward lower salt consumption on health grounds and milder tasting condiments. These trends have created pressure on manufacturers to reduce the concentrations of acid and salt, thereby reducing the inherent stability of these products.

In many cases, the spoilage boundaries for these products are not well defined, and product developers are forced to rely upon ad hoc challenge testing to give them the assurance of stability that they need. Results of challenge tests cannot easily be extrapolated to accommodate the effect of formulation changes and, thus, the rate of product innovation is constrained. A more efficient approach is to define clearly the boundaries for growth of the most significant spoilage organisms to create a space in which the product developer can work freely.

Z. bailii is acid resistant and is capable of growth in broth systems at pH values as low as 2.9 (acidified with acetic acid) or 2.2 (acidified with citric acid) *(8)*. *Z. bailii* is also considered osmotolerant *(11, 15, 16)* and, as a result, the salt and sugar levels in condiments are insufficient to control its growth by themselves. Moreover, the ability of the organism to adapt to chemical preservatives, such as potassium sorbate and sodium benzoate, is well documented *(10, 11)*. Overall, this combination of abilities makes *Z. bailii* a major concern to manufacturers of acidic condiments.

Spoilage by *Z. bailii* is likely to produce organoleptic changes in the product that may result in economic losses due to consumer complaints or recalls. On rare occasions, the evolution of CO_2 during growth of this yeast may cause a bottle to burst, creating the additional hazard of cuts from broken glass. Spoilage is most reliably prevented when the factors controlling the behavior of *Z. bailii* in the products are understood quantitatively as well as qualitatively. This can be approached effectively by modeling.

Kinetic models are useful when establishing the shelf life of perishable foods because they can help to identify the maximum time available to sell, store, and consume the

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food. Ratkowsky and Ross *(12)* pointed out that boundary models might be more useful when the primary concern relates to pathogenic organisms. Whether pathogens can grow at all and the position of the growth/no-growth boundary are of more interest than their growth rate because any growth implies a potential to multiply to an infectious dose or toxic level and to cause harm to the consumer. The same argument applies to the spoilage of foods that are distributed at ambient temperature and that require a long shelf life. These so-called shelf-stable foods are sold, stored, and consumed over long periods of time. Therefore, the ability of spoilage organisms to grow at all implies that they have the potential to multiply to sufficient numbers to cause spoilage. This could have serious commercial consequences.

This paper presents models for the spoilage boundary associated with *Z. bailii* in acidic condiments and sauces. The models are tools that can help to provide a more rational approach to product development, risk assessment, and development of sanitation guidelines than has been possible with the traditional challenge study approach. From the outset, the work was designed to establish the spoilage boundary, and experimental conditions were chosen to give a good balance between growth and no-growth conditions. All of the growth and no-growth responses were included in the statistical analyses. This approach is quite distinct from that used in kinetic modeling, where the aim is to be able to predict the time in which a particular amount of growth will occur and only those conditions where growth does occur can be included in the statistical analysis.

MATERIALS AND METHODS

Yeast strains and preparation of inocula. A laboratory strain of *Z. bailii* from the Nabisco culture collection (strain number 4637, history unknown) was used for this study. The medium used to prepare inocula for modeling experiments was yeast nitrogen broth (YNB; catalog no. 0392-15, Difco Laboratories) modified to contain a final concentration of 1% acetic acid (J. T. Baker Chemical Co., Phillipsburg, N.J.), 2% sodium chloride (J. T. Baker), and 7% fructose (Fluka, Milwaukee, Wis.). The modified broth was referred to as acidified yeast nitrogen broth (YNBA). YNBA was adjusted to pH 3.7 $\pm$ 0.1 using 20% (wt/vol) potassium hydroxide and was filter sterilized (Nalgene disposable filter, 0.45-μm pore size, Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). Aliquots (10 ml) were dispensed into sterile, polystyrene, round bottom, screw-cap tubes (16 by 125 mm, Falcon no. 2025, Becton Dickinson and Co., Lincoln Park, N.J.). *Z. bailii,* from a plate culture on potato dextrose agar (catalog no. 0013-17-6, Difco) containing 0.5% acetic acid, was transferred to a tube of YNBA using a sterile loop. The tube was incubated at room temperature for 48 h on a culture tube roller at a dial setting of 80 (Glas-Col Apparatus, Terre Haute, Ind.). An aliquot (0.1 ml) of the culture was transferred to sterile YNBA and was incubated as previously described. This subculture was repeated three more times. The cells were harvested by centrifuging in a Dynac Centrifuge (catalog no. 0101, Becton Dickinson) at ambient temperature for 10 min at a dial setting of 80 and were resuspended in 10 ml of sterile deionized water. The culture was adjusted to an optical density (OD) at 520 nm of >2.0 (approximately 10^8 CFU/ml) on a Perkin-Elmer 35 spectrophotometer. The culture was diluted in sterile deionized water to give a concentration of approximately 10^6 CFU/ml, and the concentration was confirmed by plating on potato dextrose agar supplemented with chlortetracycline·HCl (catalog no. C 4881, Sigma Chemical Co.) at 0.02 g/liter and chloramphenicol (catalog no. C 0378, Sigma) at 0.02 g/liter.

Preparation of growth media. The factorial design for the experiment gave a total of 81 different combinations of salt, sugar, acetic acid, and pH derived from high, medium, and low levels of each ($3 \times 3 \times 3 \times 3 = 81$). The individual levels of each variable are shown in Table 1.

The basal medium for the study was YNB. Concentrated stock solutions of each media component, including YNB, were prepared. Stock solutions were 5% (wt/vol) YNB, 75% (wt/wt) fructose, 25% (wt/wt) sodium chloride, and 40% (vol/vol) glacial acetic acid.

From the stock solutions, 10-ml volumes of each media combination were made. The volume of stock solution needed was calculated to compensate for pH adjustment and for addition of inoculum. Adjustments of pH were made with 20% (wt/vol) potassium hydroxide using a Gilmont 2.0-ml micrometer burette (Code GS 1200A, Gilmont Instruments, Barrington, Ill.). Measurements of pH were made on a Corning 140 pH meter. After pH adjustment, the media combinations were brought to volume with sterile deionized water, and the small volume changes involved had no measurable effect on the pH. Media were filter sterilized using Uniflo-13 sterile, disposable 0.2-μm pore size, 13-mm syringe filters (catalog. no. 28152-161, VWR Scientific, Bridgeport, N.J.).

Use of Bioscreen for growth measurements. Aliquots (350 μl) of each medium were transferred to individual wells on Bioscreen 100-well microtiter plates. Each well was inoculated with 50 μl of the yeast inoculum suspension to give a level in each well of 10^5 CFU/ml. Three replicate plates were made, each containing all 81 media combinations and one uninoculated control. The Bioscreen plates were incubated at 30°C (incubator model 1925, VWR Scientific) in closed Tupperware containers lined with moist paper towels. The volume of the containers was large, relative to the volume of inoculated broth, so buildup of volatiles and CO_2 between readings was assumed to be negligible. OD measurements for all wells were made at specified intervals up to 29 days using the Bioscreen C Analyzer (Labsytems and Affinity Sensors, Franklin, Mass.). Results were transferred electronically from the Bioscreen ASCII file to a Microsoft Excel spreadsheet for initial analysis and then to Statistical Analysis Systems for modeling using the LIFEREG procedure.

Determination of growth. The 243 (81×3) zero-time readings had a mean OD of 0.16, a standard deviation of 0.025, and a range from 0.12 to 0.21. The baseline of OD values drifted up slightly while the experiment was in progress, to a maximum of 0.25 OD in the no-growth conditions. Accordingly, the value of 0.3 OD was chosen to define growth because it was the lowest OD value that was clearly above the baseline. Linear interpolation of the OD data was used to determine a time to growth (TTG) for each of the Bioscreen plate wells. For instance, if OD measurements for days 14 and 21 were 0.15 and 0.40, respectively, the time corresponding to an OD of 0.30 would be given by $14 + [(0.3 - 0.15)/(0.4 - 0.15)](21 - 14) = 18.2$ days. In instances of no growth, TTG was censored at the final read point of 29 days. These TTG data, along with a censoring indicator and the fructose, NaCl, pH, and acetic acid levels, were the input data for model building.

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TABLE 1. *TTG from three replicate wells of each of the different salt, sugar, pH, and acetic acid levels used as the starting data set for model building*

Sugar (% wt/vol)			7.0	7.0	7.0	19.5	19.5	19.5	32.0	32.0	32.0
Salt (% wt/vol)			2.6	3.4	4.2	2.6	3.4	4.2	2.6	3.4	4.2
Maps to total molar concentration			0.83	0.97	1.11	1.53	1.66	1.80	2.22	2.36	2.50
pH	Total acetic acid (% vol/vol)	Maps to undissociated acetic acid (% vol/vol)	TTG (days)								
3.5	2.8	2.65	>29	>29	>29	>29	>29	>29	>29	>29	>29
			>29	>29	>29	>29	>29	>29	>29	>29	>29
			>29	>29	>29	>29	>29	>29	>29	>29	>29
3.75	2.8	2.55	14.3	>29	>29	>29	>29	>29	>29	>29	>29
			7.3	>29	>29	>29	>29	>29	>29	>29	>29
			17.3	24.4	>29	>29	>29	>29	>29	>29	>29
4.0	2.8	2.38	2.2	10.5	14.9	>29	>29	>29	>29	>29	>29
			2.4	2.6	8.9	>29	>29	>29	>29	>29	>29
			2.2	8.1	23.0	>29	>29	>29	>29	>29	>29
3.5	2.3	2.18	3.3	2.1	>29	>29	>29	>29	>29	>29	>29
			3.8	2.2	>29	>29	>29	>29	>29	>29	>29
			3.4	8.6	>29	>29	>29	>29	>29	>29	>29
3.75	2.3	2.09	0.9	1.5	>29	>29	>29	>29	>29	>29	>29
			1.7	2.2	2.7	12.5	22.6	>29	>29	>29	>29
			1.0	2.5	9.4	8.5	>29	>29	>29	>29	>29
4.0	2.3	1.95	0.4	0.8	1.9	5.4	>29	>29	>29	>29	>29
			0.4	1.0	1.3	3.9	>29	>29	>29	>29	>29
			0.4	0.7	2.1	>29	>29	>29	>29	>29	>29
3.5	1.8	1.7	0.3	0.5	0.9	3.4	>29	>29	>29	>29	>29
			0.4	0.4	0.7	3.5	7.8	>29	>29	>29	>29
			0.3	0.6	1.1	4.2	>29	>29	>29	>29	>29
3.75	1.8	1.64	0.2	0.7	0.6	2.3	6.1	>29	>29	>29	>29
			0.3	0.5	0.4	2.3	4.2	7.2	>29	>29	>29
			0.2	0.6	0.5	2.8	5.4	>29	>29	>29	>29
4.0	1.8	1.53	0.1	0.3	0.3	1.9	1.9	3.5	6.1	>29	>29
			0.2	0.3	0.3	1.6	2.2	3.7	5.7	>29	>29
			0.2	0.2	0.4	1.5	2.3	6.5	10.4	>29	>29

TABLE 2. *Parameters of Model 1 for TTG in terms of combined molar concentrations of salt and sugar and undissociated acetic acid*

Variable[a]	Degrees of freedom	Estimate	Standard error	Chi-square	P^b
Intercept	1	4.5290	0.2867	249.24	0.0001
s	1	3.2941	0.2627	157.21	0.0001
u	1	2.9427	0.2922	101.40	0.0001
u^2	1	0.3553	0.1162	9.34	0.0022
su	1	0.8230	0.2272	13.12	0.0003
Scale	1	0.3910	0.0347		

[a] *s*, combined molar concentration of salt and sugar; *u*, percent (vol/vol) undissociated acetic acid.

[b] Only variables with *P* values of <0.005 were included in the model.

Statistical analysis. The Statistical Analysis Systems LIFEREG procedure (SAS Institute Inc., Cary, N.C.) was used to develop predictive models of ln TTG as a function of the factors fructose, NaCl, pH, and acetic acid level. By default, the procedure fits a model to the ln of the dependent variable. The resulting model can be easily transformed to a regular time scale. Since, in growth modeling, there are often conditions for which no growth occurs, TTG is sometimes censored. Under these circumstances, ordinary least-squares regression is not applicable and special procedures are required. The LIFEREG procedure can accommodate such censored data and uses maximum-likelihood estimation methods to find regression coefficients.

Statistical modeling is an iterative process. Our strategy was to start with an all-inclusive general model and then remove noninfluential factors. The final model would hopefully be parsimonious and, yet, highly predictive. Initial models included all four main effects (percent [wt/wt] fructose = *f*, percent [wt/wt] NaCl = *n*, pH = *p*, percent [vol/vol] acetic acid = *a*), their quadratic

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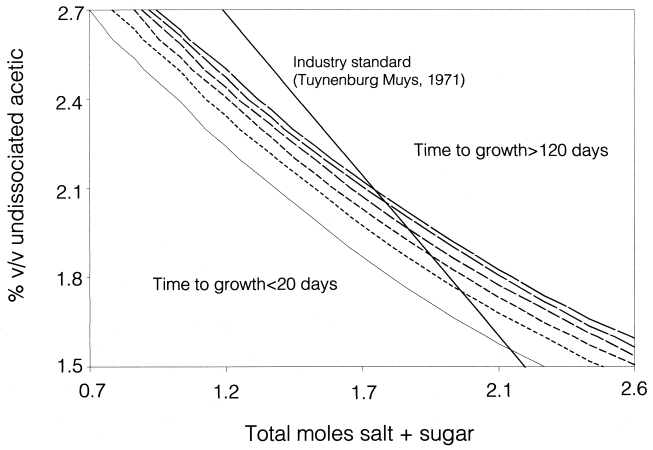

FIGURE 1. *Predicted TTG from Model 1 based on combined molar concentrations of salt, sugar, and undissociated acetic acid (percent [wt/vol]). The contours are scaled in intervals of 20 days, from 20 to 120 days, for relevance and convenience of illustration. When moving up from the growth in the <20-days region, the first contour encountered is the 20-day line. When moving down from the growth in the >120-days region, the first contour encountered is the 120-day line. The region above the 120-day contour is not a plateau but is a series of ever-increasing contours.*

effects ($f{\cdot}f = f^2$, $n{\cdot}n = n^2$, $p{\cdot}p = p^2$, $a{\cdot}a = a^2$), and the six two-factor interactions ($f{\cdot}n = fn$, $f{\cdot}p = fp$, $f{\cdot}a = fa$, $n{\cdot}p = np$, $n{\cdot}a = na$, $p{\cdot}a = pa$). These main effects, quadratic effects, and two-factor interactions are collectively referred to as factors in the ensuing. The LIFEREG procedure outputs a table of regression coefficient estimates and approximate chi-square distribution *P* values for each factor in the model. The relative importance of each factor can be judged by the *P* value: factors with small *P* values are most influential and predictive of the TTG. Final models included only those factors with *P* values less than 0.005.

The LIFEREG procedure allows the user to specify an error distribution to account for the variation in TTG not explained by the regression model. For model development purposes, Weibull, lognormal, and log-logistic distributions were considered. It was found that all three distributions gave very similar models; the same factors were retained in the final model and the final regression coefficients were similar in magnitude. Since the three distributions are not all from the same class of distributions, it is not possible to formally test for goodness of fit using likelihood ratio tests. However, since all models produced similar regression equations, we selected the log-logistic distribution model because it had the largest sample log likelihood.

Product validation. Five commercial acid condiments from the steak sauce and barbecue categories were purchased in retail stores. The products were analyzed for salt by a potentiometric method *(2),* for total sugar by high-pressure liquid chromatography *(3),* for acetic acid concentration by titration *(1),* and for pH by direct measurement using a pH meter. The products were inoculated with *Z. bailii* at a level of approximately 10^4 CFU/g and were incubated at 30°C. Samples were assessed at weekly intervals for visible gas, fermented odor, and viable yeast count determined by plating on potato dextrose agar supplemented with chlortetracycline·HCl at 0.02 g/liter and chloramphenicol at 0.02 g/liter. The products were considered to have failed this challenge test when the yeast count had increased by 1 log, visible gas bubbles had appeared, or the product smelled noticeably fermented, whichever occurred first.

TABLE 3. *Parameters of Model 2 for TTG in terms of molar concentrations of salt, sugar, and acetic acid and pH*

Variable[a]	Degrees of freedom	Estimate	Standard error	Chi-square	*P*[b]
Intercept	1	4.7573	0.2324	418.99	0.0001
f	1	3.7865	0.2379	253.31	0.0001
n	1	1.4673	0.1322	123.22	0.0001
p	1	−1.6176	0.1382	137.09	0.0001
a	1	3.2097	0.2511	163.35	0.0001
fn	1	0.5911	0.1217	23.59	0.0001
fp	1	−0.4411	0.1195	13.62	0.0002
fa	1	0.8215	0.2248	13.35	0.0003
np	1	−0.2353	0.0757	9.66	0.0019
na	1	0.4568	0.0912	25.09	0.0001
pa	1	−0.7353	0.1056	48.51	0.0001
a^2	1	0.3938	0.1216	10.48	0.0012
Scale	1	0.2728	0.2310		

[a] *f,* fructose; *n,* salt; *p,* pH; *a,* acetic acid.
[b] Only variables with *P* values of <0.005 were included in the model.

RESULTS

Growth responses. The OD measurements made by the Bioscreen showed that *Z. bailii* growth was fastest and the maximum population levels were highest in media with the lowest salt plus sugar and undissociated acetic acid concentrations. Growth was increasingly inhibited as concentrations of salt, sugar, and/or undissociated acetic acid increased. Complete inhibition over the 29-day study occurred at the highest level of undissociated acetic acid (2.65%), irrespective of the salt plus sugar molar concentration, and at the two highest molar concentrations of salt plus sugar (2.36 and 2.50 M), regardless of the amount of undissociated acetic acid (Table 1). Increasing the salt plus sugar concentration allowed a given amount of growth inhibition to be achieved at lower undissociated acetic acid concentrations, and vice versa. For example, at 0.97 M salt plus sugar, growth was inhibited at 2.65% undissociated acetic acid. By increasing the salt plus sugar to 1.53 M, growth inhibition was achieved at a lower level of undissociated acetic acid (2.18%).

Model 1. TTG based on two factors. To facilitate comparison to Tuynenburg Muys *(17),* a predictive model based on molar salt plus sugar concentration (*s*) and percent undissociated acetic acid (*u*) was developed. This was achieved by first collapsing the four-dimensional (*f, n, p, a*) space to two-dimensional (*s, u*) space by using the following relationships:

$$s \equiv \textit{moles salt} + \textit{moles sugar} = 10\left(\frac{n}{58.44}\right) + 10\left(\frac{f}{180.16}\right)$$

where 58.44 is the molecular weight of NaCl and 180.16 is the molecular weight of fructose, and

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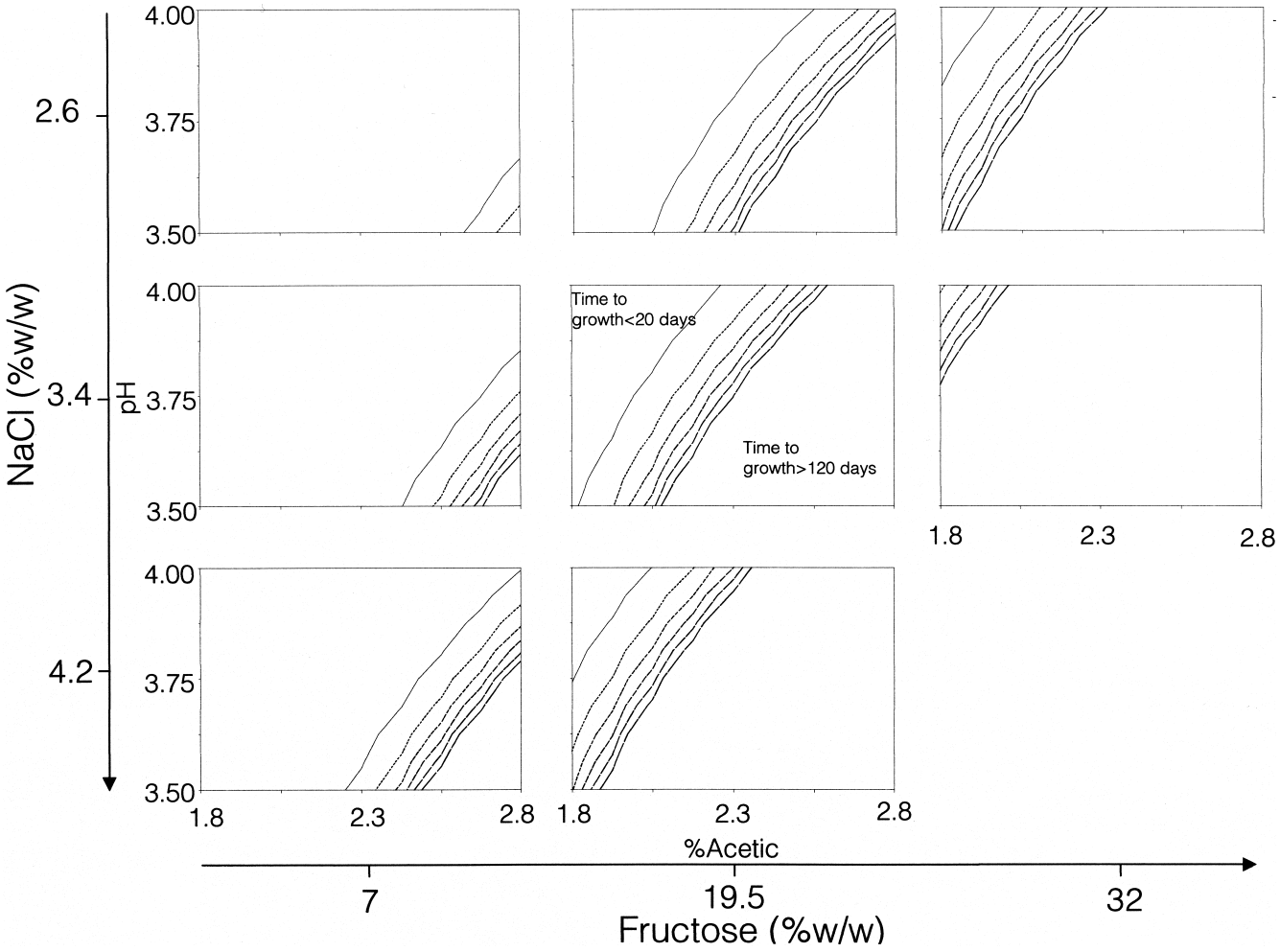

FIGURE 2. *The relationship between pH, acetic acid concentration, and TTG of* Z. bailii *at 30°C at different concentrations of salt and sugar, as predicted by Model 2. The contours are scaled in intervals of 20 days, from 20 to 120 days, for relevance and convenience of illustration. When moving up from the growth in the <20-days region, the first contour encountered is the 20-day line. When moving down from the growth in the >120-days region, the first contour encountered is the 120-day line. The region above the 120-day contour is not a plateau but is a series of ever-increasing contours.*

$$u \equiv \%(vol/vol)undissociated\ acetic = a\left(\frac{10^{-p}}{10^{-p} + 10^{-4.75}}\right)$$

where 4.75 is the pK_a of acetic acid.

These relationships were used to compute the values shown in Table 1. The TTG from the original data set (Table 1) was then used to develop a predictive model, as described above. The final model was found to be

$$\ln T = 4.53 + 3.29s + 2.94u + 0.36u^2 + 0.82su$$

where T denotes TTG. Note that s and u are normalized by subtracting their respective means and then dividing by their standard deviations. For instance, there are nine distinct values of salt plus sugar ranging from 0.83 to 2.50. These nine values have a mean and standard deviation of 1.66 and 0.6, respectively. Similarly, the nine values of percent undissociated acetic acid have a mean and standard deviation of 2.08 and 0.4, respectively. Normalized values are given by $s \equiv$ (moles salt + moles sugar − 1.66)/0.6 and $u \equiv$ (% undissociated acetic acid − 2.08)/0.4. This transformation eases numerical computation and also makes the regression coefficients more comparable.

The parameters of Model 1 are shown in Table 2. The predicted days to growth for all conditions are shown as contours in Figure 1. The predictions make good biological sense, with predicted days to growth increasing with the concentrations of salt, sugar, or undissociated acetic acid. The contours lie more closely together as the conditions become more extreme, clearly demonstrating the rapid decline in growth potential as the conditions approach the limits permitting growth. The boundary for *Z. bailii* in mayonnaise, previously determined by Tuynenburg Muys *(17),* is superimposed on the contour plot and corresponds well with the data and model generated in this study.

Model 2. TTG based on four factors. The second model is based on the original four-dimensional (*f, n, p, a*) space. The final model was found to be

$$\ln T = 4.76 + 3.79f + 1.47n - 1.62p + 3.21a + 0.59fn - 0.44fp + 0.82fa - 0.24np + 0.46na - 0.74pa + 0.39a^2$$

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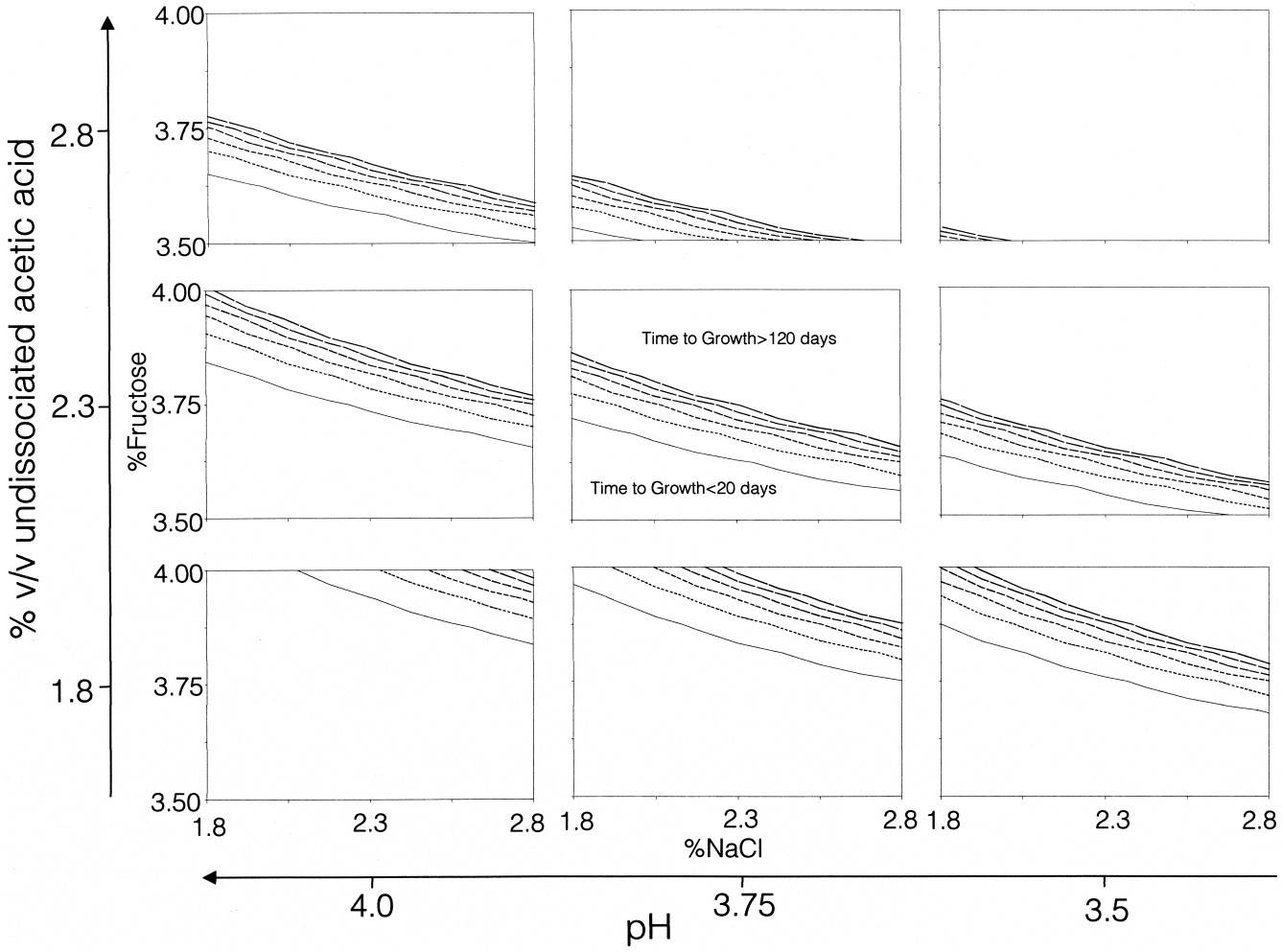

FIGURE 3. *The relationship between salt and sugar concentrations and TTG of* Z. bailii *at 30°C at different pH values and acetic acid concentrations, as predicted by Model 2. The contours are scaled in intervals of 20 days, from 20 to 120 days, for relevance and convenience of illustration. When moving up from the growth in the <20-days region, the first contour encountered is the 20-day line. When moving down from the growth in the >120-days region, the first contour encountered is the 120-day line. The region above the 120-day contour is not a plateau but is a series of ever-increasing contours.*

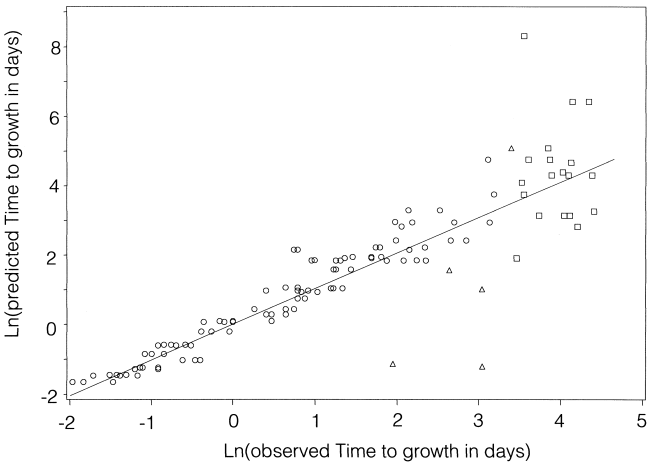

FIGURE 4. *Plot of ln-predicted against ln-observed TTG for* Z. bailii *at 30°C showing the data used to build the model (○), the data obtained for growth in media that occurred between 29 and 84 days (□), and the data for five products from the U.S. market. (△). Predictions were made using Model 2.*

The quantities (*f*, *n*, *p*, *a*) were normalized, as described above.

Model 2 predicts growth based on salt, sugar, and acetic acid concentrations and pH, all varying independently. The parameters are shown in Table 3 and predictions are shown in Figures 2 and 3. The predictions make good biological sense; in all cases, the contours become closer together and the TTG becomes longer as the conditions become more inhibitory. In Figure 2, the boundary for growth against pH and acetic acid concentration becomes more restrictive as salt or sugar concentration increases. There is a very large shift in the boundary between the lowest and highest salt and sugar concentrations, consistent with the observations of growth and no-growth conditions shown in Table 1. A similar effect is seen in Figure 3, where the boundary for growth against salt and sugar concentrations becomes more restrictive as the pH decreases or the acetic acid concentration increases.

Figure 4 shows ln-predicted versus ln-observed TTG for the data used to develop the model, the media data for growth that occurred after the end of the model-building experiment, and the data from product validation studies.

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TABLE 4. *Proportion of wells showing growth at 29 days in three replicate wells of each of the different salt, sugar, pH, and acetic acid levels used as the starting data set for model building*

Sugar (% wt/vol)			7.0	7.0	7.0	19.5	19.5	19.5	32.0	32.0	32.0
Salt (% wt/vol)			2.6	3.4	4.2	2.6	3.4	4.2	2.6	3.4	4.2
Maps to total molar concentration			0.83	0.97	1.11	1.53	1.66	1.80	2.22	2.36	2.50
pH	Total acetic acid (% vol/vol)	Maps to undissociated acetic acid (% vol/vol)	Proportion of replicates positive at 29 days								
3.5	2.8	2.65	0	0	0	0	0	0	0	0	0
3.75	2.8	2.55	3	1	0	0	0	0	0	0	0
4.0	2.8	2.38	3	3	3	0	0	0	0	0	0
3.5	2.3	2.18	3	3	0	0	0	0	1	0	0
3.75	2.3	2.09	3	3	2	2	1	0	0	0	0
4.0	2.3	1.95	3	3	3	3	0	0	0	0	0
3.5	1.8	1.70	3	3	3	3	1	0	0	0	0
3.75	1.8	1.64	3	3	3	3	3	1	0	0	0
4.0	1.8	1.53	3	3	3	3	3	3	3	0	0

This is a diagnostic test of the performance of the model. All of the points would lie on the line of equivalence where ln-predicted is equal to ln-observed TTG if the model were able to predict perfectly. In practice, this is never achieved. The figure does show generally good agreement between ln-observed and ln-predicted values for media, even for the conditions outside the range of TTGs on which the model was based. There was an increase in variability at more inhibitory conditions, and this is reflected in the increased scatter in Figure 4 at longer TTGs. The plot also shows that ln-observed TTG in products was in line with, or longer than, ln-predicted TTG. This is desirable because it shows that the model is conservative, giving fail-safe responses. These may be due to factors outside the scope of the model, for example added preservatives.

Model 3. Probability of growth. Models 1 and 2 describe the response TTG. An alternative way of characterizing growth behavior is to report whether or not growth has occurred at a particular point in time. Such data are binary (i.e., there is either growth or no growth for each test cell) and are inherently less informative than TTG data. Binary data are appropriate for modeling the response probability of growth as a function of design factors. The TTG data can be easily modified for this purpose by collapsing the TTG data set to a smaller data set, giving the number of failures observed at 29 days for each distinct set of design factors. For example, the test condition in which fructose = 7.0%, NaCl = 3.4%, pH = 3.75, and acetic acid = 2.3% had the TTG values 1.5, 2.2, and 2.5 days. At the 29-day read point, all three cells exceeded the OD limit of 0.3 and, so, were deemed to have experienced growth. For the test condition in which fructose = 7.0%, NaCl = 3.4%, pH = 3.75, and acetic acid = 2.8%, the TTG data were 24.4, >29, and >29 days. Therefore, growth occurred in just one of three test cells. Table 4 gives the number of wells with growth for all of the conditions. The LIFEREG procedure can be used to model probability of growth but, due to the binary form of the data, an additional transformation step is necessary to express the result as a probability. The final model was found to be

$$w = -3.46 - 8.99f - 3.25n + 3.77p - 6.25a$$

The result w is then transformed, as follows, to get a predicted probability

$$P(\textit{growth at } 29 \textit{ days}) = \frac{e^w}{1 + e^w}$$

Initially, all 14 effects (4 main, 4 quadratic, and 6 interactions) were fitted to the collapsed data. Not all were significant, and the final model has only main effects (Table 5). This is a consequence of the use of binary data in developing Model 3. Such data are less informative and, therefore, estimates of regression coefficients are less precise (i.e., they have higher standard errors). In fact, for these data, the larger standard errors of the regression coefficients for the second order effects in-

TABLE 5. *Parameters of Model 3 for probability of growth at 29 days in terms of molar concentrations of salt, sugar, and acetic acid and pH*

Variable[a]	Degrees of freedom	Estimate	Standard error	Chi-square	P^b
Intercept	1	−3.4564	0.7287	22.50	0.0001
f	1	−8.9945	1.7646	25.98	0.0001
n	1	−3.2533	0.7352	19.58	0.0001
p	1	3.7671	0.8124	21.50	0.0001
a	1	−6.2557	1.2539	24.89	0.0001

[a] *f*, fructose; *n*, salt; *p*, pH; *a*, acetic acid.
[b] Only variables with *P* values of <0.005 were included in the model.

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crease their *P* values above the threshold for inclusion in our final model.

Model 3 is, therefore, the simplest model of the three, having no interactive terms and predicting behavior simply in terms of the underlying factors (salt, sugar, pH, and acetic acid). The shape of contour plots based on the output from Model 3 nevertheless appeared similar to those from Model 2. This is because the quadratic and interactive terms in Model 2 are small relative to the main effects; hence, the curvature in the contours from Model 2 is small. The key difference is that the output from Model 3 gives the probability of growth at 29 days rather than the TTG.

For diagnostic purposes, plots of residual (ln-predicted and ln-observed TTG) versus main effects were examined for all three models. No unusual patterns or anomalies were seen.

DISCUSSION

Some models for growth of *Z. bailii* have been published *(4, 6, 11),* but none have studied the effects of varying the concentrations of acetic acid, salt, pH, and sugar on this organism, as was done in this study.

The models developed in this study broadly established boundaries for growth or no growth of *Z. bailii* in high-acid foods (Figs. 1 through 3). The simplest model (Model 1) is directly comparable to the Tuynenburg Muys boundary that still forms the basis of the industry standard for stability of acidic sauces *(5).* As such, the broadly defined regions of stability and instability that it generates provide guidelines for product development and illustrate the practical value of the model.

More value is to be found in Model 2, which allows the underlying factors of salt, sugar, and acetic acid concentrations and pH to be manipulated independently. This allows product developers more flexibility to adjust product composition and flavor while identifying the stability that they require. It is also important because of potential synergies between factors. For example, Cole and Keenan *(4)* showed a strong synergy between pH, water activity (°Brix), and sorbic acid on growth of *Z. bailii.* Deak and Beuchat *(6)* showed an interactive effect of water activity with pH, temperature, and sorbate on *Z. bailii.* Formulating products in the no-growth region may be constrained by acceptable limits for product consistency and/or flavor. In the unstable region, the weakest formulations can be easily identified, allowing a more informed decision on whether to reject the formula or to proceed using supplementary practices, such as kill steps, preservatives, or increased levels of sanitation.

The models may also be used as screening or investigative tools, for instance to learn about the potential stability of products of a similar type, as in the following example. Ten acidified sauces and dressings from the steak sauce and barbecue categories in the U.S. market were chemically analyzed for salt *(2),* sugar *(3),* acetic acid *(1),* and pH by direct measurement with a pH meter. Of these, only three were clearly in the stable region predicted by Model 1, based on their salt, sugar, and acetic acid concentrations and their pH. Five of the remaining seven products offered between one and three of the following additional barriers to spoilage: a declared preservative, an instruction to refrigerate the product, or a vacuum in the container indicating that the product had been hot filled. Only two were not clearly stable based on salt, sugar, acetic acid, and pH and offered no identifiable additional barriers. Commercial stability of these products could result from good sanitation practices or simply from chance that they have not been challenged by a suitably adapted *Z. bailii* strain.

Model 3 indicates the probability of spoilage (after 29 days) for any combination of the four underlying factors. This model can be used in risk assessment, where it can help to guide commercial decisions based on a known acceptable spoilage rate. One potential application would be for formulations close to the no-growth boundary. Here, decisions on whether to use chemical preservatives or to modify the underlying formula can have a significant impact on cost and flavor.

The models presented here are applicable to high-acid foods where they can be used with confidence. An additional level of utility could be achieved in the future by expanding the models to include the effect of popular food preservatives.

ACKNOWLEDGMENT

The authors thank Anthony Carella for his assistance with the statistical analyses done for this work.

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