Growth limits of *Staphylococcus aureus* as a function of temperature, acetic acid, NaCl concentration, and inoculum level

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Summary

Staphylococcus aureus is one of the most prevalent causes of gastroenteritis worldwide. Knowing the precise boundary for the growth/no growth interface of *S. aureus* and also determining the period of time needed for bacterial growth initiation is necessary for food safety risk assessment. This study was designed to examine the combined effects of temperature, acetic acid, inoculum level and NaCl concentration on the growth of *S. aureus* in brain heart infusion broth. Growth was monitored by visible turbidity over a 20 days period. Statistical analysis of data showed significant effects for selected parameters on growth of *S. aureus*. Stepwise multiple regression was used to predict the growth initiation (R^2 = 0.91, P<0.0001). To obtain a boundary model, logistic regression was used. The models accurately predicted the growth initiation and inhibition of *S. aureus*.

Key words: *Staphylococcus aureus*, Predictive model, Food safety

Introduction

Foodborne staphylococcal poisoning, caused by the ingestion of one or more preformed toxins in food contaminated with *Staphylococcus aureus*, is one of the most prevalent causes of gastroenteritis worldwide (Stewart *et al*., 2002). Outbreaks of foodborne illnesses by *S. aureus* are often associated with unsanitary handling of food at an inappropriate temperature for a prolonged period of time (Huang *et al*., 2001).

Many factors can affect the growth of foodborne microbial pathogens. Investigation the effects of environmental conditions on the growth of foodborne pathogens is crucial to control and limit their potential risks (Huang *et al*., 2001). Predictive modeling has been applied in food microbiology to describe the growth behavior of specific pathogens.

Microbial growth models are typically developed when the objective is to understand the responses of microorganisms when part of the range of conditions studied permits growth to occur. Such models can describe the increase in numbers with time (kinetic models), the conditions allowing growth or no growth (boundary models), or the chance of growth (probabilistic models) (Stewart *et al*., 2002).

Tienungoon *et al*. (2000) has divided the microbial growth models into kinetic models and probability models. The former is used to calculate the microbiological life of food products or the period of time during which the number of microorganisms in the food is less than a specified value, and with the latter, one determines whether a microorganism can grow and identifies storage conditions with a low or nil

probability of growth.

Kinetic and probability models may be closely related, because the probability of detectable growth within a specified time period depends on germination, lag, and generation times which are kinetic parameters. In some cases, a probability model may be derived from a kinetic model by some simple mathematical transformations (Tienungoon *et al*., 2000).

Application of microbial growth models to predict the growth behavior of specific microorganisms has been widely studied in recent years (McMeekin *et al*., 1997; Razavilar and Genigeorgis, 1998; Oscar, 1999; Masana and Baranyi, 2000; Zhao *et al*., 2002; Akhoondzadeh-Basti and Razavilar, 2004; Legan *et al*., 2004; Sofos *et al*., 2004; Valero *et al*., 2006). The growth/no growth interface models were first reported by Ratkowsky and Ross (1995). These models have been used by Lanciotti *et al*. (2001) for *Salmonella enteritidis*, *Bacillus cereus* and *S. aureus* as a function of water activity (a_w), pH, temperature and ethanol concentration.

In this study, we modeled the growth of *S. aureus* in brain heart infusion (BHI) broth to determine the effects of temperature, pH, sodium chloride concentration and inoculum level.

Materials and Methods

Experimental design

To assess the effects of acetic acid with different pH levels (pH), sodium chloride concentration (NaCl), inoculum level of bacteria (IL) and temperature (Temp) on growth initiation of S. *aureus*, the experiment was arranged in a factorial design in BHI broth (Merck). This design included three levels of pH (4, 5 and 7) adjusted by acetic acid, seven levels of NaCl (8, 10, 12, 14, 16, 18 and 20%), two levels of inoculum (1 and 0.1 ml of fully grown inoculated BHI broth, equivalent to 1.2×10^9 and 1.2×10^8 cfu/ml, respectively) and three storage temperatures (37°C, 25°C and 4°C) during 20 days.

Test organism

S. aureus ATCC: 9144 (Mast International Inc-England) was used as the test organism.

Preparation of inocula

The reference bacteria were plated on nutrient agar plates and incubated at 37°C for 24 h. Inoculums were prepared by transferring a loop full of the bacterial colonies to BHI broth media followed by 24 h incubation at 37°C. A second subculture was prepared by incubation for 24 h at 37°C, in a 13 × 100 mm sterile cuvett, the broth culture was adjusted to absorbance of 0.02 at 600 nm using a spectrophotometer (Jenway 6105, Essex, England). This adjustment gave a cell concentration of 1.2×10^9 cfu/ml. The number of cells in the suspension was estimated by duplicate plating from 10-fold serial dilutions on BHI agar and counting the colonies after 24 h of incubation at 37°C.

Performing the experiment

BHI powder (3.7 g) was dissolved in 90 ml distilled water in a 250 ml flask by mild heating. After cooling, NaCl was added and dissolved in amounts that satisfy the experimental design, and then the pH was adjusted using acetic acid (1 N) to designated pH values. The final volume was brought to 100 ml with additional distilled water. The values of pH were adjusted using a pH meter (Jenway Ltd., UK). The contents of each flask were dispensed into screw cap tubes at level of 10 ml per tube and then autoclaved at 121°C for 15 min. After cooling, the pH of each considered combination was measured and adjusted again with 1 N filtered-sterilized acetic acid.

S. aureus culture media (OD = 0.02 at 600 nm) was prepared and for each designated pH level a set of 42 tubes were considered for combination of different NaCl concentrations, inoculum levels and incubation temperatures. During incubation (20-day), if there was a visible increase in the turbidity of the broth, indicating the growth initiation, it was recorded and the average of the records for each single combination was calculated.

Statistical analysis

The statistical analysis was performed using SAS statistical software (version 8.2). Logistic regression was used to select the

best model to predict the growth/no growth boundary of *S. aureus* and stepwise multiple regression to predict the number of days needed for initiation of bacterial growth.

Results

All of the study parameters had significant effects on the growth initiation of *S. aureus* ($P<0.05$). The growth/no growth boundary model was completely in agreement with the observed data.

Figs. 1a to 1f show the days of visible growth initiation at different NaCl concentrations with three levels of pH, in different inoculums levels and incubation temperatures.

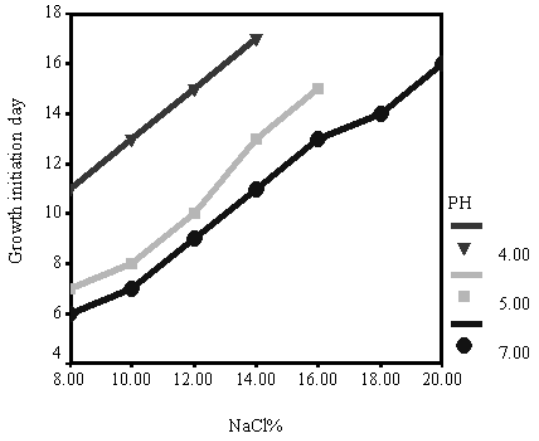

Fig. 1a: Days needed for growth initiation at different NaCl concentration by three level of pH. Inoculum level of 0.1 ml and temperature of 4ºC

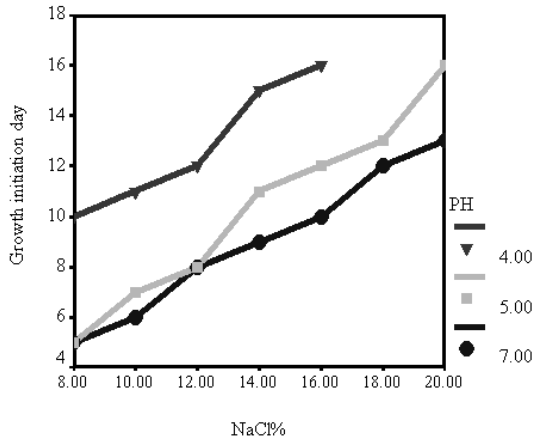

Fig. 1b: Days needed for growth initiation at different NaCl concentration by three level of pH. Inoculum level of 1 ml and temperature of 4ºC

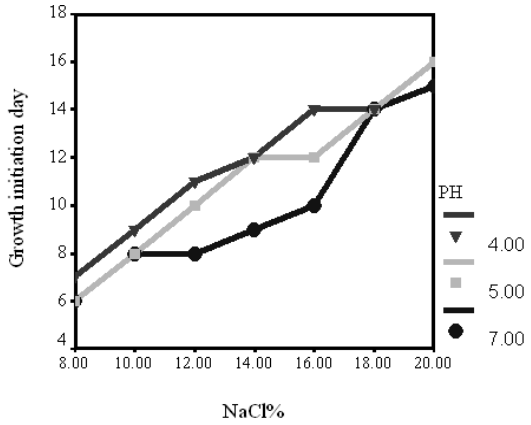

Fig. 1c: Days needed for growth initiation at different NaCl concentration by three level of pH. Inoculum level of 0.1 ml and temperature of 25ºC

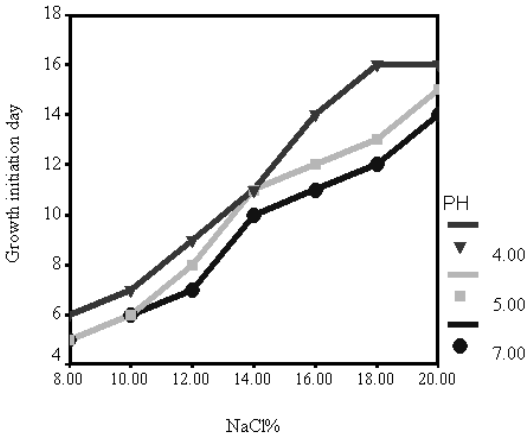

Fig. 1d: Days needed for growth initiation at different NaCl concentration by three level of pH. Inoculum level of 1 ml and temperature of 25ºC

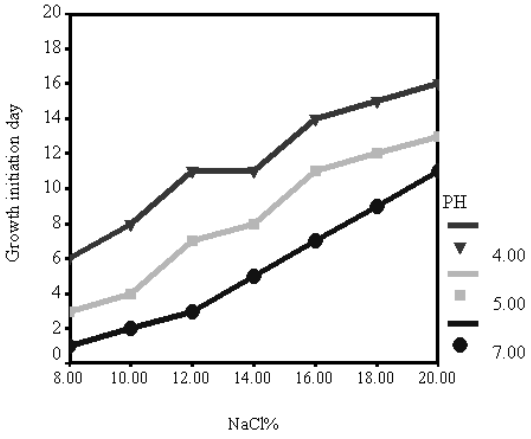

Fig. 1e: Days needed for growth initiation at different NaCl concentration by three level of pH. Inoculum level of 0.1 ml and temperature of 37ºC

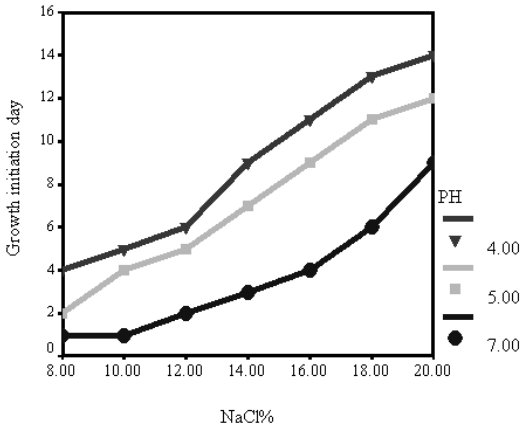

Fig. 1f: Days needed for growth initiation at different NaCl concentration by three level of pH. Inoculum level of 1 ml and temperature of 37ºC

According to Fig. 1a, the combination of 8% NaCl, pH level of 4 and IL of 0.1 ml at 4°C, the growth initiation occurred after eleven days of incubation. With increasing NaCl concentration, the duration time for growth initiation increased with a constant slope, but with NaCl ≥ 16%, the growth initiation did not occur and the broth culture media remained clear during the experiment. The same conditions were recorded at pH level of 5, IL of 0.1, at 4°C with NaCl ≥ 18%, at pH level of 4, IL of 1, at 4°C with NaCl ≥ 18%, and also at pH level of 4, IL of 0.1, at 25°C with NaCl ≥ 20%. The duration time for growth initiation was the least at pH level of 7, IL of 1, at 37°C with NaCl concentration = 8%.

For obtaining a boundary model, the logistic regression (PROC LOGISTIC in SAS version 8.2) was used to select the best model to predict the growth/no growth boundary of *S. aureus* associated with the variation of pH, incubation temperature, NaCl concentration and inoculation level. The dummy variables were created for predictor variables as ordinal numbers. The statistical results of the logistic regression model are shown in Table 1. The outcome was considered as 1 for growth and 0 for no-growth. The model equation was as follows:

Y (growth/no growth) = - [2.5399 + (2.2 NaCl) - (3.52 Temp) - (2.76 IL) - (3.25 pH)]

In this equation, a positive Y value indicates that the growth will occur, and a negative Y value suggests that the growth will not happen.

Table 1: The results of logistic regression model to predict the growth/no growth boundary of *S. aureus*

Variable	Parameter estimate	Standard error	P-value
Intercept	2.5399	2.4792	
pH	-3.2489	0.9626	0.0007
IL	-2.7646	1.1132	0.0130
Temp	-3.5220	1.083	0.0005
NaCl	2.1981	0.6004	0.0003

Another model was considered with the same dummy variables to obtain a kinetic model by the use of stepwise multiple regression (PROC REG in SAS version). The model was used to predict the growth initiation of *S. aureus* as affected by the same predictor variables. The outcome was considered as the number of days needed for initiation of bacterial growth. The no-growth samples were considered as missing values. The statistical results of the multiple regression model are shown in Table 2

TTD (time to detection) = 14.88 + 1.63 NaCl - 2.15 pH - 1.615 IL - 2.3 Temp

Table 2: The results of multiple regression model to predict the number of days needed for initiation of *S. aureus* growth

Variable	Parameter estimate	Standard error	P-value
Intercept	14.87678	0.56898	
pH	-2.14957	0.13764	<0.0001
IL	-1.61488	0.22226	<0.0001
Temp	-2.29875	0.13983	<0.0001
NaCl	1.62699	0.05884	<0.0001

$R^2 = 0.91$

Fig. 2 shows the relationship between the observed and predicted data by predictive model for time to detection (TTD) of *S. aureus* in designated combinations.

Discussion

Predictive microbiology combines mathematical modeling with experimental data on combinations of factors that influence the growth of food spoilage and/or foodborne pathogenic microorganisms. The models developed are intended to predict the fate of microorganisms in foods

(Tienungoon *et al*., 2000).

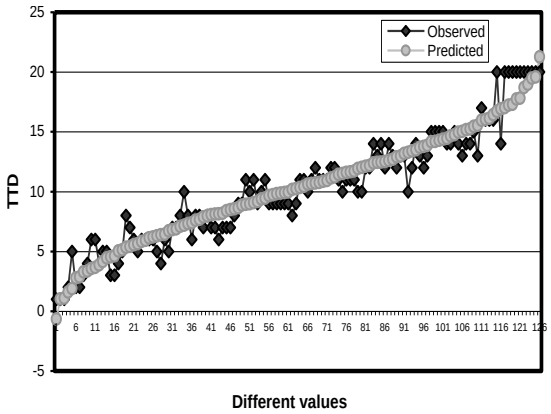

Fig. 2: Plots of observed and predicted days needed for growth initiation of *S. aureus* (TTD) according to the kinetic model

Schaffner and Labuza (1997) determined the most suitable combinations of factors in order to stop microbial growth, thus giving a significant degree of safety from spoilage or foodborne disease; this was also the aim of the hurdle approach proposed by Leistner (1985). In the present study, we determined different conditions which can stop *S. aureus* growth initiation (Figs. 1a-f) and by using logistic regression, we designed a model which can predict growth/no growth of *S. aureus* in other conditions.

In this study, three levels of pH were induced using acetic acid, an organic acid with a relatively high pKa. Environmental pH values can affect the bacterial growth. Previous models are limited to modeling the effects of pH (Rosso *et al*., 1995) not the acidulent, and thus are unable to describe the situation with foods such as meat, mayonnaise, or fermented products, which contained organic acids.

A linear response of growth rate to hydrogen ion concentration has been reported (Cole *et al*., 1990; Ross, 1993), and we observed similar response of growth limitation to acetic acid concentration (Figs. 1a-f).

We investigated the growth behavior of *S. aureus* which is a highly salt tolerant bacteria with competitive advantage on low a_w foods and has been reported to grow at a_w as low as 85% in NaCl concentrations up to 25% (International Commission on Microbiological Specifications for Foods, 1996), but in our study under optimal conditions (pH = 4, temperature = 4°C, IL = 1.2×10^8 cfu/ml) no growth was recorded, even with 12% NaCl concentration. This result is according to multiple hurdle conception in food systems.

Similar to the pH effect on microbial growth behavior, which depends on the acidulent, several authors have reported that the growth boundaries for various genera of microorganisms differ depending on the type of humectant used to depress a_w rather than the absolute value of a_w (Scott, 1957; Marshall *et al*., 1971; Christian, 1981; Chirife, 1994).

We used NaCl as humectant to depress the a_w in this model. Microbial survival and/or growth depends not only on the a_w but also on the chemical and physical properties of the humectant (Stewart *et al*., 2002).

We used two levels of inoculation. The growth of *S. aureus* was significantly affected by the inoculum size. Recent studies have indicated the importance of inoculum size on the ability of a microbial population to initiate growth (Razavilar and Genigeorgis, 1998; Masana and Baranyi, 2000; Pascual *et al*., 2001; Robinson *et al*., 2001) or the location of the growth/no growth boundary for different strains of bacteria (Robinson *et al*., 2001).

Another selected parameter was incubation temperature. The lower temperature limit for microbial growth is the temperature at which an organism is no longer able to supply the maintenance requirements (Nedwell, 1999). *Staphylococcus aureus* is a mesophilic bacterium and the temperature is one of the most important growth controlling factor such as pH and water activity that affects the location of the growth/no growth boundaries (Lanciotti *et al*., 2001; Fujikawa and Morozumi, 2006). In other experiments, decreasing the storage temperature significantly inhibited the growth rate of *S. aureus* in different foods (Lindgvist *et al*., 2002; Wong *et al*., 2004; Rho and Schaffner, 2007). These findings are in agreement with our results (Figs. 1a-f).

Probably the most important effect of temperature on the growth of a microorganism is on the shape of enzymes

required for metabolism and they will have the proper shape only within a relatively narrow range of temperatures.

Growth/no growth boundary models may be important for establishing food safety regulations. They could predict the most suitable combinations of factors to stop microbial growth, thus giving a significant degree of safety from spoilage or foodborne disease.

Since the experimental data are usually derived from studies using laboratory media, the models must be validated with data collected under conditions which food products are customarily stored (Tienungoon *et al*., 2000).

Our designated models adequately predicted the growth initiation time and growth inhibition conditions of *S. aureus* as affected by different levels of pH, temperature, NaCl concentration and inoculum level (Fig. 2).

To enhance the accuracy of the designated models, it is necessary to extend the duration of the experiment, performing the experiment with other variants and also in a wide range of selected parameters.

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