Food Sci. Biotechnol. Vol. 15, No. 5, pp. 773 ~ 777 (2006)

Food Science and Biotechnology

Estimation of Shelf Life Distribution of Seasoned Soybean Sprouts Using the Probability of *Bacillus cereus* Contamination and Growth

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Abstract Growth of *Bacillus cereus* was assessed during the storage of seasoned soybean sprouts at 0, 5, 10, and 15°C. No lag time in its growth curve was observed and thus the specific growth rate of *B. cereus* in the exponential growth phase was estimated for bootstrapped microbial count data. The distribution of the specific growth rate could be explained by the BetaGeneral distribution function, and temperature dependence was described by the Ratkowsky square root model. The temperature dependence of the growth could be successfully incorporated into the differential equation of microbial growth to predict the *B. cereus* count on the seasoned soybean sprouts under fluctuating temperature conditions. Safe shelf lives with different probabilities to reach 10^5 CFU/g were presented at four different temperatures, considering the variation in initial contamination and specific growth rate by the Monte Carlo method and 2-step bootstrapping, respectively. Safe shelf lives defined as the time with a probability of less than 0.1% of reaching the critical limit, were 13.4, 5.2, 3.6, and 2.8 days at 0, 5, 10, and 15°C, respectively.

Keywords: microbial growth model, *Bacillus cereus*, bootstrap, Monte Carlo method, temperature

Introduction

Many Korean side dishes are prepared by seasoning the blanched vegetables with dry spices and other ingredients (1). Nowadays these side dishes are increasingly prepared in food processing facilities, then distributed and marketed under chilled conditions. This trend is in accordance with the growth of the cook-chilled food market worldwide (2, 3). Microbial criteria have been reported to be an important quality index for chill-stored Korean seasoned side dishes, and as such may be used universally to determine shelf life (4). One type of bacteria that is of concern in the safety of these chill-stored prepared foods is *Bacillus cereus*, which may cause spoilage and food-borne disease (3, 5-8). *B. cereus* is a ubiquitous, psychrotrophic, and heat-resistant bacterium that can grow at minimum temperatures of 4-10°C and is found in soil, cereals, raw vegetables, and spices (8, 9). Its psychrotrophic properties combined with its heat-resistant nature make it a potential hazard for refrigerated storage of Korean prepared foods.

Seasoned soybean sprouts are a typical product of cooked and chill-stored side dishes in Korean market. They are prepared by mixing blanched soybean sprouts and various dry spices, so have a potential risk of *B. cereus* contamination. In one study, *B. cereus* strains were found in 12 out of 17 soybean sprout samples (10). Control of the microbial risk and shelf life of seasoned soybean sprouts based on the growth of *B. cereus* will help to ensure the safe delivery of this product to consumers. Since there are variations in the initial microbial contamination level and in the growth characteristics of the microbial strains, the risk assessment and shelf life determination must take these factors into account.

This study was aimed at developing a method to determine the shelf life of Korean seasoned soybean sprouts, based on the growth kinetics of *B. cereus* at various temperatures. The time taken to reach a certain level of microbial growth was defined as the shelf life and this was determined for different combinations of initial contamination levels and storage conditions. Variability in microbial growth and contamination level was taken into account in the estimation of the shelf life.

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Received July 8, 2006; accepted August 29, 2006

Materials and Methods

Seasoned soybean sprouts Seasoned soybean sprouts were purchased in the summer season directly from a local preparation shop in Masan, Korea. The product consisted of 24 g salt, 19 g minced garlic, 18 g sesame oil, and 8 g sesame salt powder for each 1,000 g of steamed soybean sprouts. A preliminary experiment in the summer season confirmed that the seasoned sprouts used in the experiment contained *B. cereus*. The product was transferred to the laboratory immediately after preparation and was used for the storage experiment.

Storage of soybean sprouts at constant and fluctuating temperature conditions First, the seasoned soybean sprouts were stored under constant temperature conditions of 0, 5, 10, or 15°C. Seasoned soybean sprouts were placed in rectangular 18.0×13.0×2.0 cm polystyrene trays, 100 g sprouts/tray. All the trays were covered with 12 μm thick linear low density polyethylene (LLDPE) cling film. Periodically during the storage period, three trays were taken to measure the *B. cereus* count of the product. Plate counting was conducted in triplicate for each tray, to give nine replicate microbial counts for a storage time test.

Secondly, time-variant temperature conditions were

applied to trays of seasoned soybean sprouts. During the storage period, 10 g of seasoned soybean sprouts were aseptically removed from a tray to measure the *B. cereus* count of the product.

***B. cereus* counts** For determination of *B. cereus* counts in soybean sprouts, 10 or 20 g of the sample were aseptically transferred into sterile Stomacher bags and diluted with 40 mL of sterile 0.1% peptone water. Samples were then homogenized in a Stomacher blender (Lab-Blender, TMC International, Seoul, Korea) at 300 strokes/min for 2 min and aliquots of 0.1 mL were plated out directly or as 10-fold dilutions in 0.1% peptone water on mannitol-egg yolk-polymixin (MYP) Agar CM0929 (Oxoid, Basingstoke, Hampshire, England) supplemented with *B. cereus* selective supplement SR99 (Oxoid) and egg yolk emulsion SR47 (Oxoid). Plate counting was performed in triplicate. After incubation of 72 hr at 30°C, pink colonies surrounded by a precipitation zone were counted as *B. cereus* colonies and expressed as colony-forming units (CFU) per gram (11).

Parameter estimation of *B. cereus* growth model The exponential phase alone was observed in our experimental data of *B. cereus* growth on the soybean sprouts. Processing and preliminary storage before the experiment started may have allowed the bacterial strain to adjust to the immediate growth. Therefore the specific growth rate at each storage temperature was determined from the slope of the logarithmic growth curve as given by Eq. 1.

$$\log_{10}N=\log_{10}N_o+\frac{\mu}{\ln 10}t \quad (1)$$

where, N is the *B. cereus* count in CFU/g at time t, N_o is the initial *B. cereus* density in CFU/g, and μ is the specific growth rate (1/day).

The experimental data of microbial count included experimental error and variance, so the average microbial count data during storage were obtained by bootstrapping of individual plate counts and used for estimating μ by minimization of sum of squares (12, 13). The bootstrapping method is based on the construction of bootstrap samples, which are obtained by random sampling with replacement from the original data. It generates a large number of independent bootstrap samples, each being of the same size as the original data. An estimate of any statistic can be obtained from the bootstrap replications (13). The bootstrapping and parameter estimation in this study were repeated 1,000 times to obtain the distribution of μ. The computational algorithm was coded in Fortran language and run under the environment of Digital Visual Fortran® (Digital Equipment Corporation, Maynard, MA, USA).

Simulation of *B. cereus* growth under fluctuating temperatures For simulation of *B. cereus* growth under fluctuating temperature conditions, Eq. 2, which is the differential form of Eq. 1 for the exponential growth phase, was solved numerically by Runge-Kutta 4th order method incorporating time dependent specific growth rate of μ.

$$\frac{dN}{dt}=\mu N \quad (2)$$

Temperature dependence of μ was described by the Ratkowsky square root model, as described in Eq. 3:

$$\sqrt{\mu}=b\cdot(T-T_{min}) \quad (3)$$

where, T is the temperature (°C), b is a parameter, and T_{min} is the theoretical minimum temperature for growth estimated by extrapolation of the regression line to the temperature axis.

Shelf life determination under various conditions Shelf life at different temperatures was estimated based on the following calculation of *B. cereus* growth as derived from Eq. 1:

$$t_s=\frac{\ln 10\cdot\log_{10}N_c/N_o}{\mu} \quad (4)$$

where, N_c is the critical limit of 10^5 CFU/g. The limit of 10^5 CFU/g is the critical level generally accepted for *B. cereus* in USA and most European countries (14-16).

The variability in initial contamination level was taken into account using the Monte Carlo method on its experimentally determined distribution: lognomal distribution with mean of 0.72 and standard deviation of 1.10. Out of the 18 samples taken in the summer season, 14 samples were under the detection limit for *B. cereus* of 1.5 CFU/g, and thus a de-rounding process was applied to complement the data for those samples by 1.5R, where R is a random number generated between 0 and 1 (17). The specific growth rate at any storage temperature was taken by bootstrapping the 1,000 specific growth rate values estimated from bootstrapped microbial data as described above. This was considered to be the variability in growth rate of *B. cereus*. The calculation of microbial growth-based shelf life was repeated 10,000 times for randomized Monte Carlo selection of initial contamination level and bootstrapped parameter estimation. The range of safe shelf life was estimated by examining the distribution of the time taken for the bacterial count to reach the critical level of 10^5 CFU/g.

Results and Discussion

Microbial growth model parameters Figure 1 shows the average counts of *B. cereus* on seasoned soybean sprouts, which were stored under different temperature conditions. For all four temperature conditions, no lag time was observed. Lag phase is strongly dependent on the history and the physiological state of the microorganisms (18), and absence of the lag time in the growth curve suggests that the processing conditions of the seasoned soybean sprouts in the summer may have allowed the immediate adaptation and growth of the contaminated *B. cereus* strain. The conditions of the initial sample and preparation with dry spices seemed to have made the bacteria ready for exponential growth. Delignette-Muller and Rosso (14) also analyzed the growth of *B. cereus* without consideration of the lag phase. The assumption of lag phase absence is taken as a worst-case scenario for microbial risk assessment.

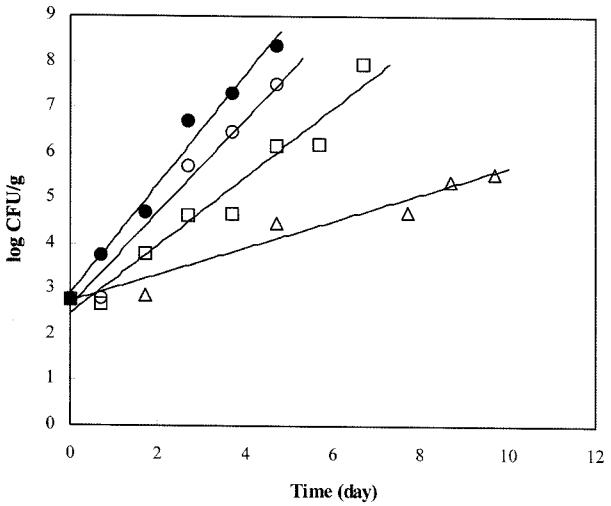

Fig. 1. Counts of *B. cereus* on seasoned soybean sprouts stored at 0, 5, 10, and 15°C. Solid lines are the estimations by Eq. 1 or 2 with average μ. Each microbial growth datum is the average of nine plate counts. △, 0°C; □, 5°C; ○, 10°C; ●, 15°C.

Using the bootstrapping method for individual plate counts, the model parameter of μ was determined 1,000 times in order to find its distribution (Fig. 2). The plots in Fig. 2 overlay bootstrap distributions of specific growth rate with its probability density functions (comparison of probability density). One of several goodness-of-fit plots, it gives the most valuable information about the parameter distribution pattern. These plots offer a visual comparison between the data and fitted distributions. The probability density of μ at four temperatures can be described best by BetaGeneral distribution (Fig. 2):

$$\text{BetaGeneral}(\alpha_1,\alpha_2, \text{min}, \text{max}) = \frac{(x-\text{min})^{\alpha_1-1}(\text{max}-x)^{\alpha_2-1}}{B(\alpha_1,\alpha_2)(\text{max}-\text{min})^{\alpha_1+\alpha_2-1}}$$

where, $B(\alpha_1,\alpha_2) = \frac{\Gamma(\alpha_1)\Gamma(\alpha_2)}{\Gamma(\alpha_1+\alpha_2)}$, and α_1 and α_2 are continuous shape parameters greater than 0, and min and max are continuous boundary parameters. The fitted distribution function may be used to characterize the probability of the microbial growth parameters and give the stochastic estimation of microbial growth. BetaPert and normal distribution functions have been used in other studies to explain the variability of microbial growth rate (14, 19).

When the specific growth rate value averaged from all μ values in Fig. 2 was incorporated into Eq. 1 or 2 for integration under static temperature conditions, the predicted microbial counts followed the experimental data (Fig. 1). Temperature dependence of average μ, which is described according to the Ratkowsky equation (Eq. 3), gave a b value of 0.0582 $day^{1/2}/°C$ and a T_{min} of -14.6°C (Fig. 3).

When the differential Eq. 2 was solved with the

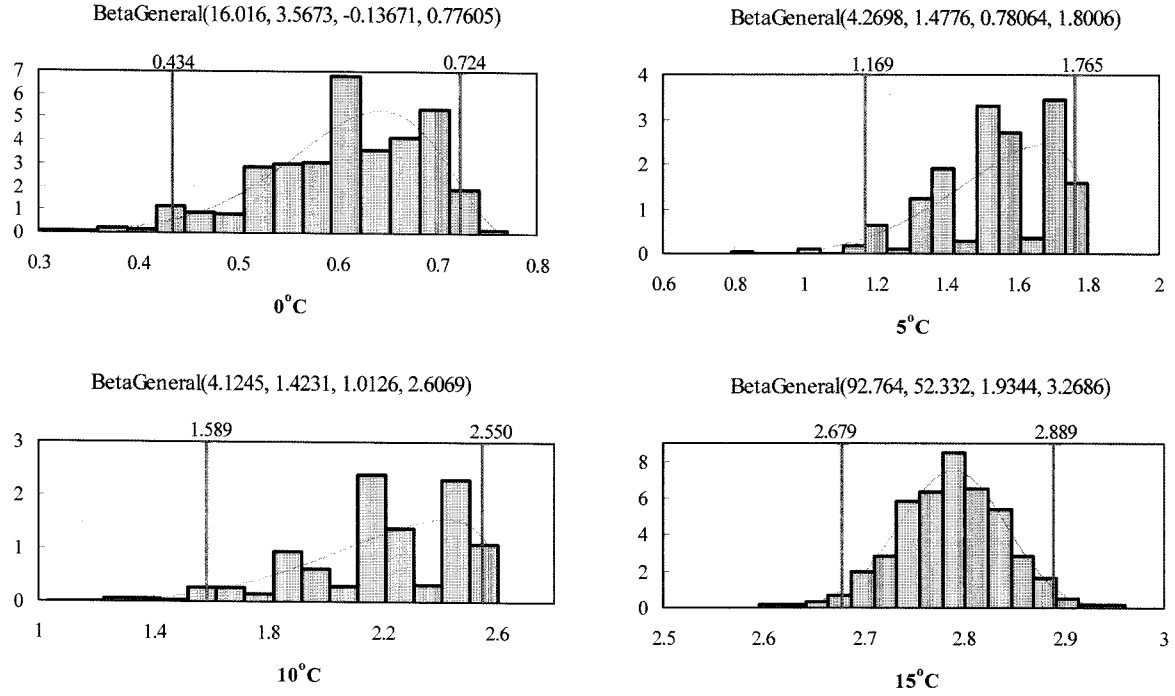

Fig. 2. Distribution of *B. cereus* specific growth rate on seasoned soybean sprouts stored at 0, 5, 10, and 15°C. Shape parameters of α_1 and α_2, and boundary parameters of min and max in the BetaGeneral distribution function $\left(\frac{(x-\text{min})^{\alpha_1-1}(\text{max}-x)^{\alpha_2-1}}{B(\alpha_1,\alpha_2)(\text{max}-\text{min})^{\alpha_1+\alpha_2-1}}\right)$ are given in brackets. The left and right vertical lines in the graph are the 2.5 and 95% quantiles of the bootstrap distribution of μ.

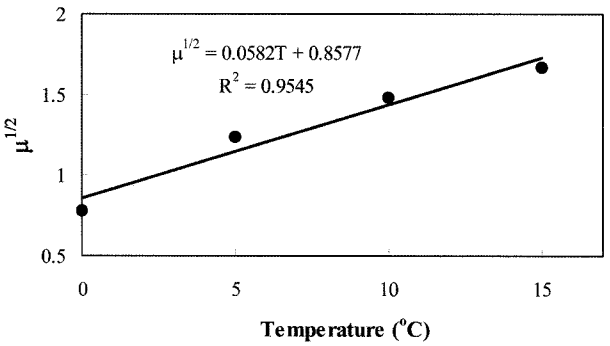

Fig. 3. Temperature dependence of the average specific growth rate.

incorporation of temperature effect on μ, *B. cereus* count under dynamic temperature conditions could be estimated to be close to the experimental data (Fig. 4). The good agreement between experimental and estimated microbial counts suggests that the growth model with estimated parameter can predict the growth of *B. cereus*, enabling it to be used for on-line shelf life estimation. The food may undergo exposure to dynamic temperature conditions during the food supply chain, so estimation of microbial growth under fluctuating temperatures is useful for real-time control of shelf life and may be combined with time temperature integrator in the future.

Estimation of shelf life at different temperatures based on *B. cereus* growth Figure 5 shows the estimated shelf life distribution at four temperatures. The time to reach 10^5 CFU/g may differ with variation in initial contamination and specific growth rate, factors that were considered in the Monte Carlo estimation of initial contamination and 2-step bootstrapping of specific growth rate, respectively. If the safe shelf life is set as the time with a probability of less than 0.1% that the critical limit of 10^5 CFU/g will be reached, the seasoned soybean sprouts can be stored for

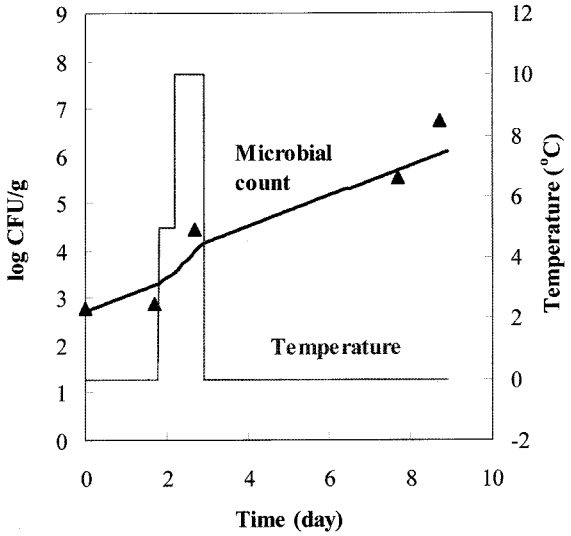

Fig. 4. Comparison between experimental and simulated *B. cereus* growth on seasoned soybean sprouts stored under dynamic temperature conditions. The thick line is the estimated *B. cereus* growth and the thin line shows the temperature change. ▲ : experimental data.

13.4, 5.2, 3.6, and 2.8 days at 0, 5, 10, and 15°C, respectively (Fig. 6). Figure 6 presents average shelf life values to reach the *B. cereus* count of 10^5 CFU/g. It should be noted that estimated shelf life distribution depends on the distribution and magnitude of both microbial contamination and specific growth rate. Therefore, the conditions of food preparation and contaminated microbial flora may change the shelf life estimation. Processing variables such as blanching conditions and mixing recipe can affect the initial contamination level, water activity, and pH, which in turn influence the microbial growth and the time to a critical level. Accurate information on the contamination level and food conditions would allow more reliable shelf life estimation and control. Sufficient accumulation of the

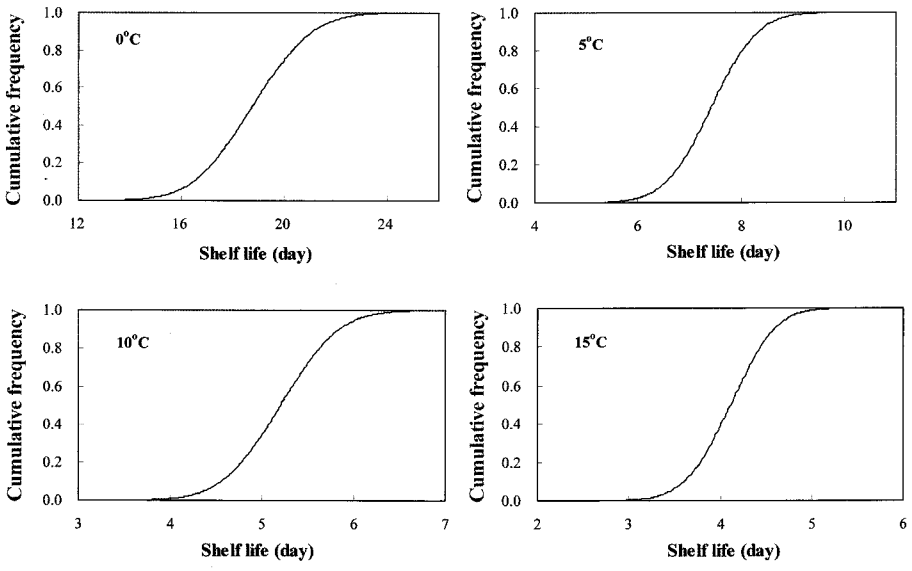

Fig. 5. Cumulative distribution of shelf life estimates for *B. cereus* count to reach 10^5 CFU/g at different temperatures.

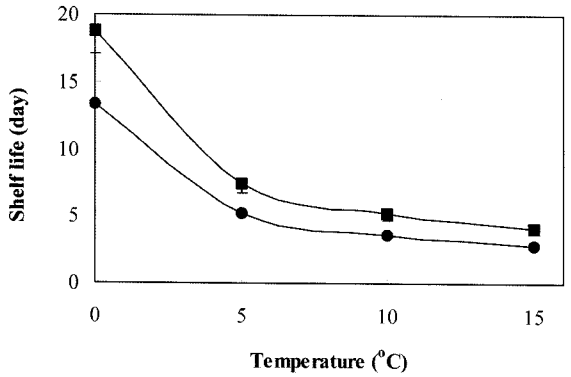

Fig. 6. Shelf life estimates with different probabilities as function of temperature. ●, value with probability less than 0.1%; ■, average. Vertical bars indicate standard deviation of the estimate values.

experimental data on *B. cereus* contamination and growth is suggested for better logistical control of this type of product.

Shelf life estimation can be based on many different criteria including chemical, sensory, and microbial quality indexes (20). Microbial quality changes in shelf life determination are concerned with food spoilage and safety. Shelf life estimation based on *B. cereus* may be seen as a guideline for safety control in food distribution. Other criteria-based shelf life determination may be used for the delivery of better quality product to consumers.

In conclusion, the parameter of specific growth rate of *B. cereus* in the exponential growth phase on seasoned soybean sprouts was estimated at four temperatures, and then its distribution and temperature dependence were examined. When the temperature dependence of the parameter was incorporated into the differential equation of microbial growth, growth of *B. cereus* on seasoned soybean sprouts could be predicted under fluctuating temperatures. Shelf lives at four different temperatures with a critical level of 10^5 CFU/g were presented taking into account the variation in initial contamination and specific growth rate, by Monte Carlo estimation and 2-step bootstrapping, respectively. This kind of shelf life estimation can be applied to other chilled foods where *B. cereus* growth is of concern.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (Project # R01-2005-000-10235-0).

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