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Optimization of heat and relative humidity conditions to reduce *Escherichia coli* O157:H7 contamination and maximize the germination of radish seeds

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ABSTRACT

We previously reported that a combination of heat and relative humidity (RH) had a marked bactericidal effect on *Escherichia coli* O157:H7 on radish seeds. Here, response surface methodology with a Box-Behnken design was used to build a model to predict reductions in *E. coli* O157:H7 populations based on three independent variables: heating temperature (55 °C, 60 °C, or 65 °C), RH (40%, 60%, and 80%), and holding time (8, 15, or 22 h). Optimum treatment conditions were selected using a desirability function. The predictive model for microbial reduction had a high regression coefficient ($R^2 = 0.97$), and the accuracy of the model was verified using validation data ($R^2 = 0.95$). Among the three variables examined, heating temperature ($P < 0.0001$) and RH ($P = 0.004$) were the most significant in terms of bacterial reduction and seed germination, respectively. The optimum conditions for microbial reduction (6.6 log reduction) determined by ridge analysis were as follows: 64.5 °C and 63.2% RH for 17.7 h. However, when both microbial reduction and germination rate were taken into consideration, the desirability function yielded optimal conditions of 65 °C and 40% RH for 8 h (6.6 log reduction in the bacterial population; 94.4% of seeds germinated). This study provides comprehensive data that improve our understanding of the effects of heating temperature, RH, and holding time on the *E. coli* O157:H7 population on radish seeds. Radish seeds can be exposed to these conditions before sprouting, which greatly increases the microbiological safety of the products.

1. Introduction

Pathogenic *Escherichia coli*, which include enterohemorrhagic bacteria, are frequently associated with catastrophic outbreaks of food poisoning after the consumption of various sprout products (Breuer et al., 2001; Buchholz et al., 2011; Mohle-Boetani et al., 2001; Watanabe et al., 1999). In 2011, sprouts contaminated by shiga toxin-producing *E. coli* O104:H4 caused 4000 cases of infection and 53 registered deaths (Uphoff et al., 2014). A multistate outbreak of *E. coli* O157:H7 caused by contaminated alfalfa sprouts occurred in the United States in 1997, resulting in four cases of hemolytic uremic syndrome in 1 month (Breuer et al., 2001). In 1996, more than 6000 primary school children in Japan were infected with *E. coli* O157:H7 after consuming contaminated sprouts (Watanabe et al., 1999). Because this pathogen is present in pre-sprouted seeds, a small number of bacteria can proliferate rapidly during the sprouting process (Beuchat, 1996; Buck et al., 2003; Stewart et al., 2001); therefore, reducing bacterial contamination on seeds is important if we are to protect public health.

To ensure the microbiological safety of sprout products, the National Advisory Committee on Microbiological Criteria for Foods recommends that treatments should result in a 5 log reduction in the pathogen population present on the seeds (NACMCF, 1999). Various methods have been tested to achieve this standard, including treatment with chlorine solution or various combinations of physical and chemical treatments (Bang et al., 2011; Fransisca et al., 2011; Neetoo and Chen, 2011; Nei et al., 2013). However, difficulties with respect to application to sprout products due to the economic and technical reasons, consumer avoidance of chemicals, and deterioration of the products after treatment remain a concern. In a previous study, we reported a novel method for eliminating *E. coli* O157:H7 from radish seeds using only heat and relative

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humidity (RH) (Kim et al., 2015). This technology is both clean and simple and does not require chemicals; as such it has potential industrial applications, as most sprout producers have their own growth chamber. We found that by manipulating the temperature and RH we could achieve a 7 log CFU/g reduction in the *E. coli* O157:H7 population on radish seeds, with no detrimental changes to the seeds themselves. However, before this technology can be introduced into sprout processing lines, a theoretical model must be developed to establish the optimal conditions.

Response surface methodology (RSM) is one of the most popular statistical methods for optimizing product processing in the food industry and is based on data obtained from appropriately designed experiments (Gupta et al., 2012; Kwak et al., 2011; Prasad et al., 2011). Using this model, independent variables can be controlled to achieve optimal responses for the production of prime products (Nwabueze, 2010). Box-Behnken design is a very useful tool for the multivariate optimization of RSM, leading to a statistical model with fewer design points than central composite designs (Ferreira et al., 2007).

Here, we investigated the effects of heating temperature, RH, and holding time on the *E. coli* O157:H7 population on radish seeds and then examined the germination rate of treated seeds. A Box-Behnken experimental design for RSM was used and a desirability function was applied to optimize these three independent factors to achieve maximal reductions in microbial contamination of seeds. The most significant factors were selected and mathematically modeled to achieve the optimum treatment conditions, which were then verified.

2. Materials and methods

2.1. Bacterial strains and cell suspensions

Three *E. coli* O157:H7 strains, ATCC 35150 (acid resistant; human feces isolate), 43889 (acid adaptable; human feces isolate), and 43890 (acid sensitive; human feces isolate) were used in this study were provided by the Food Microbiology Culture Collection at Korea University (Seoul, Korea). All strains were stored at −20 °C in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) containing glycerol (20% w/v). Before use in the experiments, an inoculum of each strain was cultured in TSB (90 ml) at 37 °C for 24 h in a shaking incubator (VS−8480S, Vision Scientific, Seoul, Korea) at 225 rpm. Cells were collected by centrifugation (3000 × g for 15 min at 4 °C) (MF80, Hanil Science Industrial Co., Ltd., Gangneung, Korea) and washed twice in 0.85% sterile saline (final cell concentration: ca. 8−9 log CFU/ml). The supernatants were then discarded.

2.2. Sample preparation and dip inoculation

The radish seeds (*Raphus sativus)* used in this study were purchased from Daenongbio (Gyeonggi-do, Korea) and stored in a refrigerator (4 °C) before inoculation. Radish seeds (90 g) were immersed in the bacterial cell suspensions (Ninety ml of each individual culture was combined to make a total volume of 270 ml). The combined culture was gently agitated with a sterilized spatula at room temperature (22 °C) for 10 min to ensure uniform distribution (7−8 log CFU bacteria/g seeds). After the inoculum was decanted, the samples were dried in a laminar-flow biosafety hood for 2 h at room temperature and stored in a refrigerator (4 °C) overnight to allow bacterial attachment.

2.3. Experimental design: response surface modeling

Three independent variables (heating temperature, RH, and holding time) affect the number of *E. coli* O157:H7 present on radish seeds. Preliminary study of independent parameters yielding an effective reduction in the *E. coli* O157:H7 population indicated that the optimal ranges for each of these factors were as follows: temperature, 55 °C, 60 °C, or 65 °C; RH, 40%, 60%, or 80%; holding time, 8, 15, or 22 h. RSM with a Box-Behnken design was used to examine the effects of each factor, and to determine the optimal treatment conditions for microbial inactivation with a minimal number of experimental runs (Gao and Jiang, 2005). Each factor was assigned a code of −1, 0, or +1 (Table 1) and the experimental design comprised 15 trials. To verify the accuracy of the model equations, the reduction in the bacterial population was assayed under 10 randomly selected treatment conditions within the range (Table 5).

2.4. Single or combined condition of heat and RH

A custom-built device containing saturated solutions of chemicals was used to generate humid conditions as previously described (Kim et al., 2015). Briefly, aqueous solutions (200 ml) of potassium carbonate (K_2CO_3; Sigma−Aldrich, St. Louis. MO, USA), lithium acetate (CH_3COOLi; Sigma−Aldrich), and potassium chloride (KCl; Sigma−Aldrich) were used to adjust the RH to 40, 60, or 80%, respectively. Aqueous solutions were equilibrated in a polypropylene container measuring 237 × 112 × 111 mm (Lock and Lock, Asan, Korea). A smaller polypropylene container measuring 120 × 86 × 52 mm (Lock and Lock) was placed inside the larger container for setting seeds. The water activity (A_w) of each solution was measured using a humidity machine (Labmaster- A_w; Novasina, Lachen, Switzerland) and values converted to RH using the following equation:

$$\text{RH } (\%) = \text{Aw} \times 100(\textit{in equilibrium})$$

Each inoculated sample was transferred to the custom-built device. Samples (25 g) were subjected to an RH of 40%, 60%, or 80% at 22 °C for 22 h, or to an RH of 40%, 60%, or 80% in combination with heat treatment at 55 °C, 60 °C, or 65 °C. The device was heated in an incubator (VS−1203P3V, Vision Scientific) and the atmospheric conditions validated using a thermo-hygrometer (YM. TL-6460; Electronics Tomorrow Ltd., Kowloon, Hong Kong) placed inside the device.

2.5. Microbiological analysis

Twenty-five grams of inoculated radish seeds were either treated or not treated and then immediately transferred to stomacher bags (Circulator 400 standard bags; Seward, Worthing, UK) containing 225 ml of 0.85% sterile saline. The bags were then stomached at 230 rpm for 2 min (Circulator 400, Seward). One milliliter of homogenate was then serially diluted 10-fold with 9 ml of 0.85% sterile saline. Next, a 0.1 ml aliquot was spread-plated onto MacConkey Sorbitol Agar (Difco, Detroit, MI, USA) in duplicate, and 0.2 ml of undiluted sample was directly plated onto each of five plates to reach the lower detection limit (detection limit = 10 CFU/g of radish seeds). The agar plates were incubated at 37 °C for 24 h and typical colorless (sorbitol negative) colonies counted. Each trial was repeated three times.

Table 1
Experimental ranges and levels of the independent variables.

Variables	Symbols	Range and levels		
		Low (−1)	Medium (0)	High (+1)
Temperature (°C)	X_1	55	60	65
RH (%)	X_2	40	60	80
Time (hours)	X_3	8	15	22