Guidelines for Process Validation Using *Enterococcus faecium* NRRL B-2354

The Almond Board of California (ABC) funded research projects with Silliker Research Center, Michigan State University, The National Food Laboratory, and Deibel Laboratories, Inc. in an attempt to identify surrogates for *Salmonella* Enteritidis Phage Type 30 (SE PT 30) for use in microbiological challenges of almond processing. After evaluating the results of those research projects, the ABC Technical Expert Review Panel (TERP) identified *Enterococcus faecium* NRRL B-2354 as a surrogate for validation of almond thermal processes. The following procedures for inoculation of almonds, sample handling, and enumeration of treated samples provide guidelines for use in validation trials for almond processing.

Surrogate Culture

The *Enterococcus faecium* strain NRRL B-2354 (labeled as *Pediococcus* sp.) used in the research projects was obtained from the National Center for Agricultural Utilization Research (NCAUR), U.S. Department of Agriculture (Dr. Alejandro P. Rooney, 1815 N. University St., Peoria, IL 61604, tel: 309-681-6395, fax: 309-681-6672, alejandro.rooney@ars.usda.gov). This same strain is also available from the American Type Culture Collection (ATCC) and is designated *Enterococcus faecium* ATCC 8459. Recently, researchers at the University of Georgia confirmed that *Pediococcus* sp. NRRL B-2354 is a strain of *Enterococcus faecium* using 16S rRNA gene sequencing techniques and a Vitek 2 microbial identification system (Ma, L., J. Food Protection, Vol. 70, No. 4, 2007, Pages 952-957). Note that NCAUR distributes this strain as *Pediococcus* sp. NRRL B-2354 without charge, while ATCC distributes it as *Enterococcus faecium* ATCC 8459 for a fee. Although the ATCC 8459 and NRRL B-2354 represent the same strain, we recommend use of the NRRL B-2354 strain from NCAUR for validation testing since the surrogate research projects used this source for the culture.

Applicability

This surrogate is applicable for use in validation studies of dry heat processes of almonds, such as dry roast, dry roast flavoring, brine and pre-wet dry roast, dry plasticizing, etc.; moist air processes such as steam plasticizing, ambient steam processes (FMC, Ventilex, Biosteam, etc.); and other alternative heating treatments such as infrared, microwave and radio frequency heating of dry or pre-wet almonds.

Inocula Preparation

- Upon receipt, the culture (fresh or frozen) should be streaked onto tryptic soy agar (TSA, Difco) and incubated at 35°C for 24+/2 hours. Isolated typical colonies should be picked and transferred into tryptic soy broth (TSB, Difco) and incubated at 35°C for 24+/2 hours. After incubation the culture should be transferred into TSB broth and incubated overnight at 35°C. Five (5), 1-ml aliquots of the overnight (18 ± 2 h) culture should be spread over each of five 150 mm x 15 mm TSA plates (5 plates for surrogate and 3 plates for SE PT 30) to produce a bacterial lawn after incubation for 24 ± 2 h at 35°C. Following incubation, approximately 5 to 6 ml of 0.1% peptone should be added to each large plate. The bacterial lawn should be loosened with a sterile spreader and a sterile pipette should be used to collect the cells (yielding a total of approximately 25 ml). Prior to inoculating the almonds, the appropriate number of 25-ml preparations (to inoculate 400-g portions of almonds)

should be pooled and thoroughly mixed for a minimum of 1 minute using a magnetic stir bar and stir plate. The inocula should be kept on the stir plate until all of the almond samples have been inoculated.

Preparation of Inoculated Almonds

- Nonpareil almonds, grade US #1 and size 27/30 should be used for inoculation. Chipped and scratched kernels should be excluded. The moisture level of the almonds must be 4.5 - 5.5% prior to inoculation. To assure the almonds have a low background bacterial load, almonds pasteurized by treatment with propylene oxide (PPO) (with no detectable PPO residue) should be used in the validation trials.
- Almond samples (400 ± 1 g) should be weighed into plastic polyethylene bags (30.5 cm x 30.5 cm), and 25 ml of the pooled inocula added. The bags should be closed and mixed by hand by repeated inversions for 1 minute. Almonds should be poured out of each bag and spread onto two sheets of 46 x 57-cm filter paper (Fisher brand Qualitative P8, Fisher Scientific, Pittsburgh, PA) that are folded in half and positioned on a metal drying rack inside a large plastic tub (Rubbermaid, Wooster, OH). Almonds should be stored for 24 hours or longer at 24 ± 2ºC with the lid ajar to let them dry. Several batches of inoculated almonds may be prepared and pooled together.

Confirmation of Inoculated Almond Batch

- The counts of the inoculated almonds should be determined after inoculation. The inoculation level must be greater than 7 logs per gram.
- The inoculated almonds should have a moisture level of less than 8.0%.
- The microorganisms on the inoculated almonds should demonstrate acceptable heat resistance when the log reduction is less than 2.5 logs for 25 grams of the inoculated almonds scattered on an aluminum mesh rack (kernels are separated) and exposed to heat treatment at 280°F for 15 minutes in a Fisher Scientific Isotemp 851F oven or equivalent device.

Handling of Inoculated Almonds

- After drying, the inoculated almonds may be stored at 38-40°F prior to heat treatments. However, the inoculated almonds must be used within 14 days. Inoculated almonds older than 14 days may be used providing they meet the requirements listed under “Initial Counts of Inoculated Almonds”.
- The inoculated samples should be conditioned to carrier product temperature prior to an actual testing run.
- On the same day that validation trials are being conducted, traveling controls must be handled in the same manner as treated samples, except for the treatment. Triplicate samples of the traveling controls should be enumerated to determine the level of microorganisms.

Heat Treatment of Inoculated Almonds in Processing Lines

- The inoculated almonds should be loosely packed in 50-gram portions in thermal-stable plastic netting or equivalent to closely mimic operating conditions of the system to be validated.
- The 50-gram sample bags may be embedded among almond kernels on the conveyor or mixed in the product flow if the operation does not utilize a conveyor. A sufficient number of sample bags must be used in each validation run to cover representative locations of the conveyor bed. The placement of the sample bags should concentrate on the coldest spots previously identified by the temperature mapping.
- Sufficient replicate runs for each parameter must be conducted to address process variations.

Enumeration of Treated Almond Samples

- After being retrieved from the processing line, the treated samples should be stored at 38-40°F and enumerated within 24 hours. If the treated samples are hot when they are retrieved, they must be placed into Whirl-Pak bags and chilled immediately in an ice bath.
- The 50-gram treated samples should be transferred to 100 ml of TSB in a 710-ml Whirl-Pak bag that has been tempered at ambient temperature for 4 hours. The samples bags should be mixed for 2 minutes using a Stomacher Lab Blender, and then held for 3-5 minutes prior to further dilution.
- Then, the sample bag should be vigorously shaken by hand 5 times followed by serial dilution in Butterfield's phosphate buffer solution (BPB).
- The enumeration procedures described in the FDA Bacteriological Analytical Manual (United States Food and Drug Administration, 1998) may be also followed. Briefly, 50 to 100-g sub-samples were added to 50 to 100 ml of Butterfield's phosphate buffer, respectively. Samples were shaken vigorously 50 times in a 30 cm arc and after standing for 5 minutes were shaken an additional five times before serial dilution and plating.
- 0.1-ml aliquots (for spread plating) should be plated in duplicate onto TSA plates that have been tempered at ambient temperature for at least 4 hours. The plates may be made the previous afternoon and left at ambient temperature overnight.
- Plates should be counted by hand 48h after incubation at 35°C following the procedures outlined for plate counting in the Compendium of Methods for the Microbiological Examination of Foods.

Report and Interpretation of Results

- All raw microbiological counts and respective log values should be tabulated together as a part of the process validation report. Log reductions may be obtained by subtracting log of survivors of a treated sample from log of counts for the corresponding untreated control. Average and minimum log reductions should be reported. The minimum log reduction should be calculated by subtracting the highest survivor log of the count from the treated samples under each parameter from the lowest log of the count from the corresponding untreated inoculated samples.
- A process or a set of parameters that has demonstrated a minimum log reduction greater than 4.0 will meet the minimum ABC treatment requirement.