HEAT RESISTANCE OF *PAENIBACILLUS* SP. SPORES ISOLATED FROM CARBONATED JUICE BEVERAGE

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ABSTRACT

Hurdle technology is the most common method employed by the food industry to control for microbial growth of both pathogens and spoilage organisms in food products over extended shelf lives. The spoilage organisms of an apple juice beverage (10% v/v) overcame several hurdles, including low pH, pasteurization, and carbonation, resulting in turbidity of the beverage. Two organisms were isolated and determined by genetic sequencing to belong to the *Paenibacillus* genus, but no specific species could be identified. *Paenibacillus* spp. are endospore forming, heat resistant, and can be acid tolerant. Thermal death curves were obtained for spores of one isolate at 85, 90 and 95 ºC. The D-values and z-value were calculated from the linear regressions of these curves. This heat resistance data may contribute to the understanding of *Paenibacillus* spp. spores as well as hurdle technology design for similar beverages.

BIOGRAPHICAL SKETCH

Sophia Elie was half raised in Port of Spain, Trinidad and the other half in Oak Park, IL. She holds a Bachelor of Science and Engineering degree in Chemical Engineering from the University of Michigan in Ann Arbor. Post undergrad, she entered the food & beverage industry. Sophia currently has 9 years of industry experience in cold fill preserved, hot fill, and aseptic beverage manufacturing facilities. Her past roles include Production Supervisor, Research & Development Quality Analyst, and Quality Engineer. She currently works in Food Safety as a Process Authority. Sophia completed this master's degree part-time while working full time as a beverage Process Authority.

To the 22-year-old Sophia who truly believed she would never attend graduate school because a graduate degree would be an unnecessary waste of time. Keep following the path of curiosity with a dose of skepticism.

ACKNOWLEDGMENTS

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INTRODUCTION

Microbial food spoilage occurs due to the growth of microorganisms (bacteria, yeasts, and molds) in food, which can cause changes to color, odor, flavor, and/or texture of products that are deemed undesirable by consumers. The food industry attempts to prevent microbial spoilage and prolong the shelf life of products. Common processes implemented to prevent microbial spoilage include high temperature processing, refrigeration or freezing, reduction of water activity, acidification, addition of protective microorganisms, and addition of chemical preservatives (e.g. benzoates, sulfites, sorbates, nitrites). However, the prevalence of cancer and other chronic diseases predominant in western societies has led to the association of these diseases with food processed for control of microbial growth, especially foods with added chemical preservatives [1]. Consequently, consumers are highly concerned about the types of foods they eat and how food affects their health. The food industry has effectively used preservatives to control for microbial spoilage of products, yet many consumers believe most preservatives are harmful to their health. As a result, there is a high demand for products with no added preservatives yet the same extended shelf-life, providing what is now referred to as a "clean label". The most common approach used to meet this demand is hurdle technology.

A clean label apple juice beverage (10% v/v) was manufactured on a commercial processing line with the applied hurdles of acidification, pasteurization, and carbonation, then stored at ambient temperature for an expected 16-week shelf life. After 8 weeks of storage, several bottles were identified as cloudy, an indication of spoilage by microorganisms that overcame the hurdle design. Further information about the spoilage organism is needed to ensure preservation of the beverage for the intended shelf-life. This project determines the spoilage organisms to be of the genus *Paenibacillus* and studies the heat resistance of its spores to modify the hurdle preservation design for the beverage and other similar products.

Hurdle technology

Previously used empirically for centuries by a vast number of cultures around the world, the "hurdle technology" concept was scientifically introduced as a tool in the early 1990s [2]. Hurdle technology is applied by most food manufacturers to ensure the microbiological safety and stability of food products by purposely combining multiple preservation methods or hurdles. Therefore, these hurdles are always product and package specific. Fundamentally, hurdle technology methods disrupt the cellular homeostasis maintained by targeted microorganisms, as the hurdles create a hostile environment for them. This disruption can keep the microorganisms in lag phase; microorganisms cannot overcome the hurdles that strain their repair mechanisms or metabolically exhaust them, which can lead to cell death [3]. A low pH hurdle, or challenge, affects proton transport across the cell membrane leading to changes in respiratory chain complexes that pump protons out of and bring protons into the cell[4]. The wet heat of pasteurization works by irreversibly denaturing the biological macromolecules essential to cell function and growth. Carbonation can provide an almost anaerobic environment that is detrimental to microorganisms that need oxygen for metabolic processes. The combined hurdles can have lethal effects on enzyme activity, protein stability, structure and function of carbohydrates, lipids, proteins, and nucleic acids within the cell. Therefore, a microbial population initially present in the food from ingredients or the manufacturing environment should not be able to overcome the usually sequentially applied hurdles.

More than 100 hurdles of choice exist; common physical hurdles can include thermal processing, storage temperature, radiation, electromagnetic energy, high pressure, ultrasonication, packaging, modified atmosphere packaging, and aseptic packaging. While physico-chemical hurdles often include changing the water activity, pH, redox potential and adding salts and nitrites. Microorganisms may overcome the hurdles if the initial microbial load

is extremely high. However, when thoughtfully designed for the initial load and types of microorganisms present, there can be synergistic effects allowing for less intense hurdles. Sometimes applied simultaneously, the multiple preservation methods can target different aspects of the microbial cell. Thus, the hurdle metaphor has since been challenged as it does not illustrate the additive or synergetic effects of hurdle technology design [5]. If an organism can overcome the designed preservation hurdles, the initial load could have been too high, intensity of the hurdles may not be high enough, or an additional hurdle is needed.

Paenibacillus spp.

A growing genus with over 293 species at the time of writing, *Paenibacillus* spp. are ubiquitous [6]. Strains have been isolated from the guts of insects, plants, and humans, and are known spoilage organisms for fluid milk. These rod-shaped, endospore-forming bacteria were once classified under the genus *Bacillus*. However, after being described as phylogenetically distinct, *Paenibacillus* became a novel genus with "*Paeni*" derived from latin meaning "almost"; an almost *Bacillus* [7]. *Paenibacillus* spp. are aerobic or facultatively anaerobic, reported to gram stain positive, negative or variable, and motile with flagella. Some species of *Paenibacillus* can solubilize phosphorous to be accessible to plant roots, promote iron uptake by plants, fix nitrogen, and others can produce antimicrobial peptides that control phtyopathogens [8]. With regard to pathogenicity, *P. larvae* is a known pathogen to honeybees, causing American foulbrood (AFB) disease, and these residual spores can be found in honey [8]. *P. glabratella* is a pathogen for snails and other *Paenibacillus* spp. can be opportunistic pathogens for humans, but usually in elderly whom have compromised immune systems [8]. Both *Bacillus* spp. and *Paenibacillus* spp. spores can survive extreme conditions including high temperature short time (HTST) processing of milk. From over 1,228 *Paenibacillus* spp. isolates obtained from dairy products and dairy environments, *P. odorifer* was the predominant species with the ability to

grow at 6°C and reduce nitrate [9]. While most *Paenibacillus* spp. grow optimally at neutral pHs, *P. terrae* was found to be acid tolerant and isolates were observed to grow at pH 5 and 7 °C. [10]. The apple juice (10% v/v) beverage spoilage suggests that *Paenibacillus* spp. spores may survive and grow in low pH and carbonated environments.

Spoiled product identification

The apple juice (10% v/v) beverage was acidified to a target pH of 3.8, then pasteurized at 203°F +/- 2.7°F for approximately 10 seconds. The product was then carbonated to a target of 3.0 +/- 0.2 gas volumes (GV), aiming for a carbonation level of 2.0 GV after 16 weeks of ambient storage in PET bottles. Post processing, the bottles were cased, palletized and held at environmental temperatures ranging from 75-80 °F, then inspected weekly. Visual spoilage was discovered at week 8, 12, and 16 with over 4,700 turbid bottles identified out of approximately 38,000 bottles. The failures were reported to be evenly distributed across all retained pallets for the entire run.

METHODS

The spoilage organisms were isolated directly from turbid bottles, prepared for PCR amplification and sequencing, cultured for spore crops, and then heat treated to determine thermal death values.

Isolation

The juice beverage was vacuum filtered using sterile 0.22 µm filters (Millipore S.A.S, France) and sterile magnetic filter funnels (Pall Life Sciences, New York). The filters were plated onto Standard Plate Count (SPC) agar, acidified potato dextrose agar (PDA), and malt extract agar (MEA), then aerobically incubated at 30°C for 48 hours. Growth only occurred on SPC plates.

DNA preparation and PCR amplification

Three separate isolates were streaked onto SPC to obtain isolated cultures and were incubated at 30°C for 48 hours. PCR amplification was completed twice, first with a 16S ribosomal RNA primer then with a rpoB primer, the β-subunit of RNA polymerase, designed for *Bacillus* and *Paenibacillus* genera (Table 1). Bacterial colonies were suspended in sterile deionized water in PCR tubes, then microwaved on high heat for 4 minutes. Lysed samples were added at 2 µL with the following concentrations for a 25 µL reaction: 0.15 units/µL GoTaq Flexi Polymerase, 1X GoTaq Green Master Mix (ProMega, Wisconsin, MI), and 0.5 mM forward / reverse primers. PCR products were analyzed with agarose gel electrophoresis and visualized using ethidium bromide before submitting for Sanger sequencing by the Cornell Biotechnology Resource Center.

TABLE 1: *Primers and thermocycling parameters for PCR amplification*

Locus	Primer	Thermocycling Parameters
16S [11] rRNA	fD1 - 5'AGAGTTTGATCCTGGCTCAG3' rD1 - 5'- AAGGAGGTGATCCAGCC-3'	94℃ 5 min 35 cycles of 94 ℃ for 30 sec 50 ℃ for 1 min & 72 ℃ for 2 min 72 ℃ for 10 minutes. PCR products kept at 4 ℃
rpoB [12] RZrpoBV3	RZrpoBRV3 – 5'- TGNARYTTRTCRTCRACCATGTG-3' RZrpoBFV3 – 5'- AARYTNGGHCCDGARGAAAT-3'	94℃ for 3 minutes 94°C for 30 sec AT* for 30 sec | 20 cycles at 55-45°, 72°C for 1min | 20 cycles at 45°C 72°C for 7 min

* the first 20 cycles have an AT that decreases by 0.5°C for each cycle (touch down PCR). Then, 20 cycles with an AT at 45°C

Spore suspension preparation

Isolates were cultured in tryptic soy broth (TSB) at 30°C for 48 hours, then 25 plates were streaked for full plate coverage on SPC agar. The spores were harvested from the plates

after 7 days of incubation at 30°C or when observed by microscopy to be mostly covered in spores. Each plate was flooded with 10 mL of sterile Butterfield's (3M, Canada) buffer, then scraped with a sterile spatula to loosen the cells from the agar. Spore suspensions obtained from the plates were pooled into a large sterile tube resulting in stable spore crops with an initial population of 10^6 to 10^7 CFU/ml. Spore suspensions were stored at 4°C until experimental use.

Heat resistance of spore suspensions

The heat resistance of the spores was determined using the capillary tube method. Using a sterile 1-ml syringe and repeater dispenser (Hamilton Co., Reno, NV), 5 sterile glass melting point capillary tubes (1.5-1.8 X 90 mm; Kimble Chase, Vineland, NJ) were filled with 40 microliters of spore suspension in buffer. Each capillary tube was flame sealed then placed into distilled water-filled test tubes in a stirred thermostat-controlled water bath. Spores were thermally treated in the water bath at temperatures of 85, 90, and 95º C. Each treatment was performed as triplicate experiments with three individual test tubes of spore suspensions simultaneously submerged in water for a specific time interval then immediately transferred to an iced ethanol bath. Serial dilutions were performed using Butterfield's phosphate buffer and surviving spores were enumerated on SPC. Plates were incubated at 30°C for 48 hours. Survival curves were fitted to a linear regression model to calculate the decimal reduction times (D-values). The z-value (temperature needed to reduce the D-value 10-fold) was calculated as the reciprocal slope of the fitted linear regression line for log of D- values versus temperature curve.

RESULTS

Sequencing Results from PCR gene amplification

Using the National Center for Biotechnology Information (NCBI)'s Basic Logic Alignment Search Tool (BLAST), the amplification of the 16S rRNA gene of these spoilage organisms was most similar to a *Paenibacillus humicus* species with 96% similarity and a

Paenibacillus ehimensis with a 95% similarity. Amplification of the rpoB gene indicated 2 distinct species. The first isolate returned a 98.6% pairwise identity for 710 base pairs as "*Paenibacillus RUD330"* submitted by a researcher of Moscow State University in 2017. The next closest match had a 91.8% pairwise identity for an unidentified *Paenibacillus* species. "*Paenibacillus RUD330"* was obtained from a freshwater city pond and is hypothesized to be a symbiont of algae *Euglena gracilis*. The species has not yet been characterized using traditional methods or validly published. The second isolate had a pairwise identity of 89% across 713 base pairs for *Paenibacillus koreensis* and similar 89-86% pairwise identity for the next eight best scoring matches.

Heat Resistance

Decimal reduction times at each temperature were calculated as the negative reciprocal of the regression line fitted to the data (log CFU/ml versus time curve). After heating the spores at 85°C for 30 minutes, an approximately 1 log reduction was achieved (Figure 1). For the same amount of heating time, about a 2-log reduction was achieved when heat treated at 90°C (Figure 2). Only about 5 minutes was needed to achieve a 2-log reduction at 95°C (Figure 3).

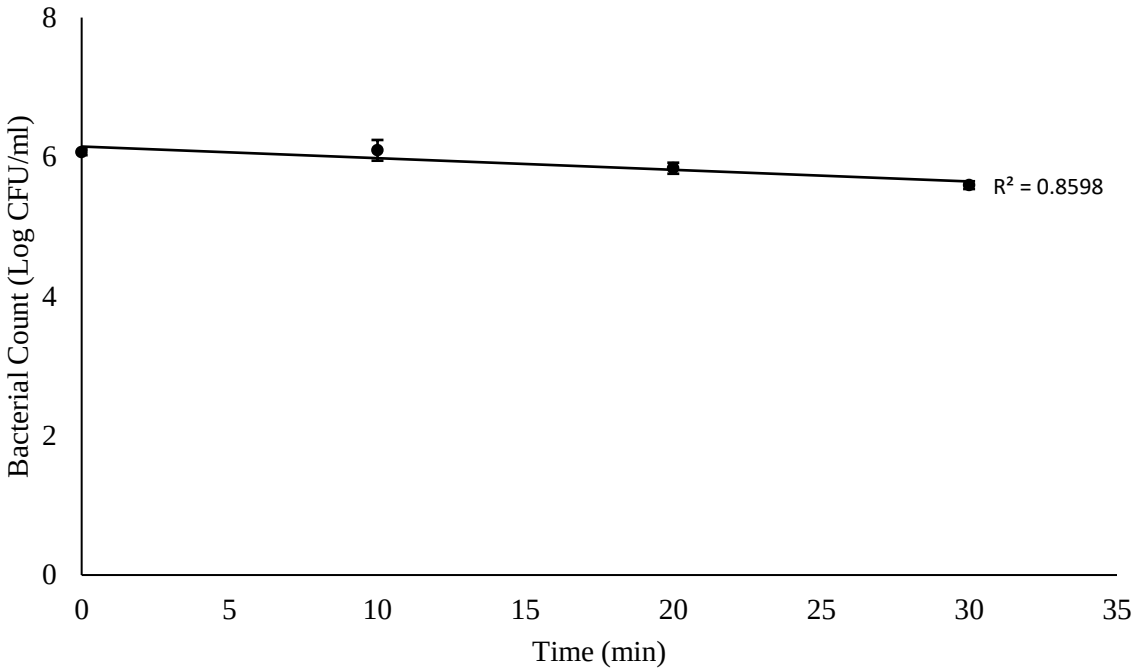

FIGURE 1. *Survival curve of spores at 85°C in Butterfield's buffer.*

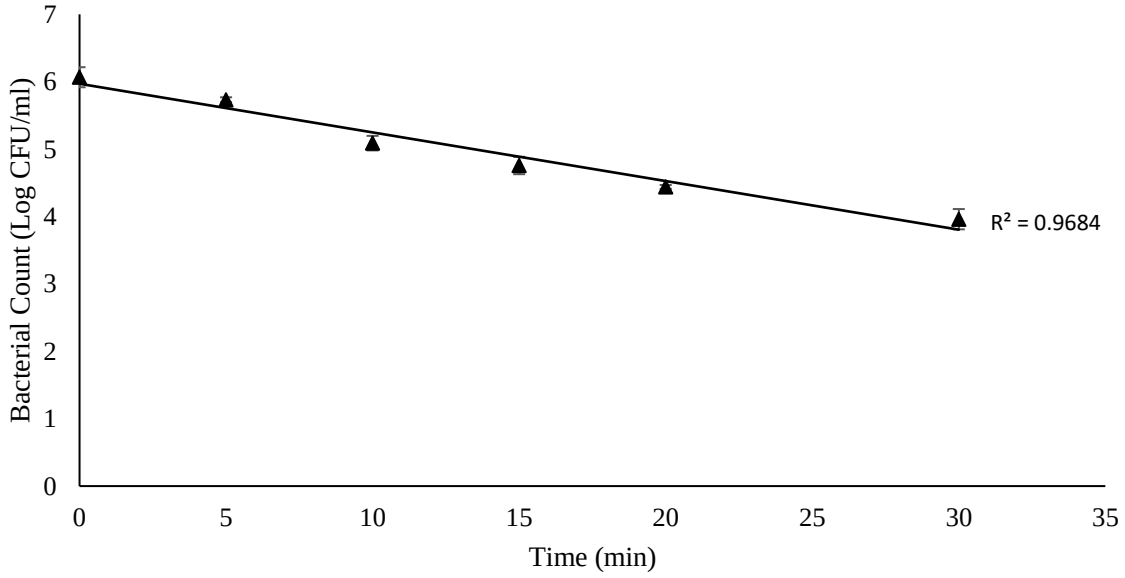

FIGURE 2. *Survival curve of spores at 90°C in Butterfield's buffer.*

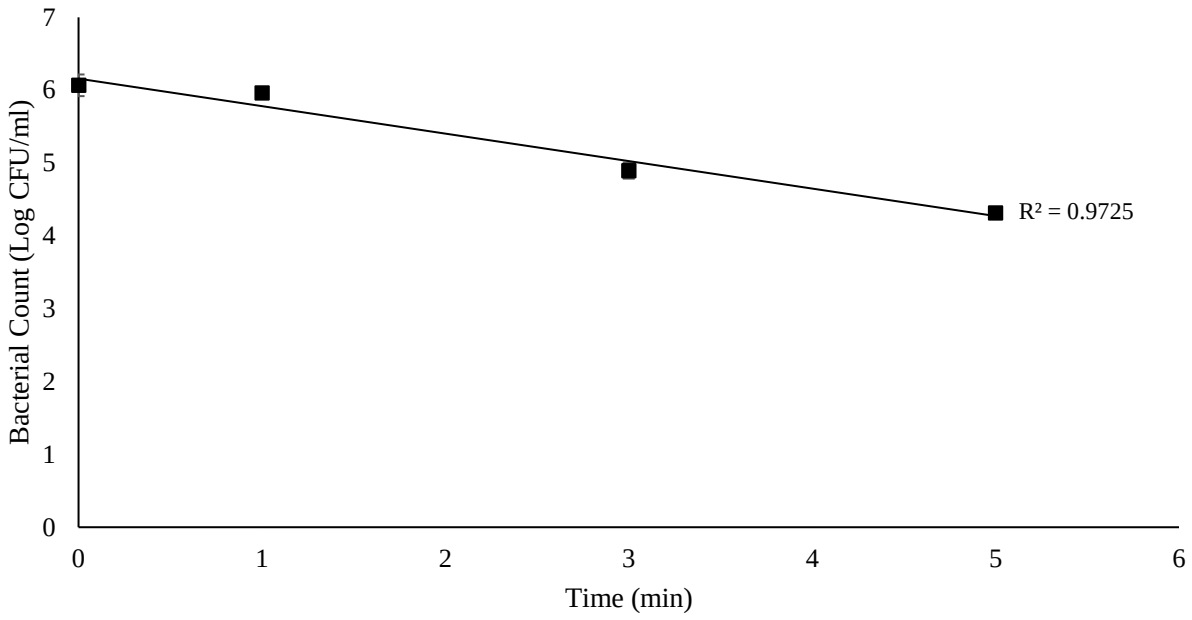

FIGURE 3. *Survival curve of spores at 95°C in Butterfield's buffer*.

The calculated D-values at 85, 90, and 95º C, are in Table 2. The z-value calculated from the negative reciprocal of the regression line fitted to the log D-value vs temperature curve is 8.35°C (Figure 4).

TABLE 2. *Decimal reduction times (D-values) of spores in Butterfield's buffer*

Temperature (°C)	**D- value (min)**
85	38.3
90	14.7
95	2.43

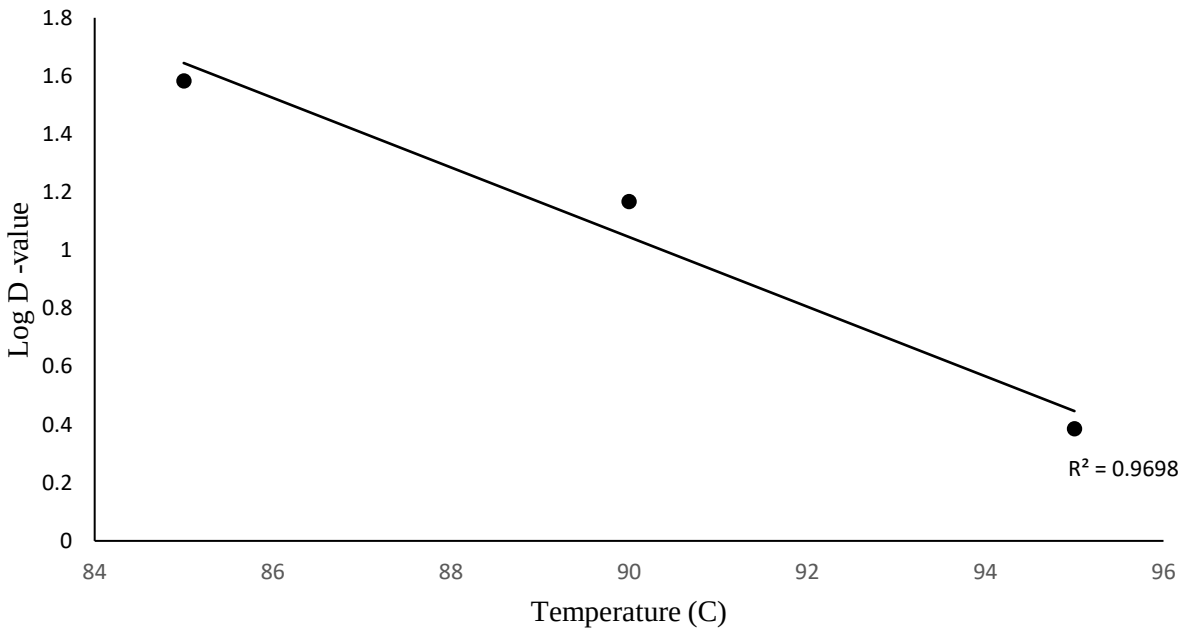

FIGURE 4. *Heat resistance curve of spores in Butterfield's buffer*

DISCUSSION

PCR amplification and sequencing results

The currently recommended sequence similarity thresholds for classifying bacterial isolates by genus and species are 95 % and 98.7 %, respectively. PCR amplification of the 16S rRNA gene only provided sequencing results with 95 -96% similarity to a few species and the thresholds should only be used as indicators rather than an absolute tool [13]. The 16S rRNA PCR amplification was only successful in identifying the genus, *Paenibacillus*. Thus, the rpoB gene was selected for PCR amplification to further determine the species. With a 98.6% sequence similarity and a large drop off in score for the other matches, the first isolate is likely "*Paenibacillus RUD330*" or a closely related species. However, as this species has not been characterized or validly published, further studies on the strain are warranted. Moreover, the second isolate was resolved only to *Paenibacillus* genus as both 16S and rpoB gene

amplification failed to return matches that were distinct enough to provide confidence. For these two potentially novel species, whole genome sequencing and biochemical characterization should be completed to confirm the identity of these taxa and their association with spoilage.

Heat resistance of Paenibacillus *spp. isolates*

In this study, 1 of 2 isolated *Paenibacillus* spp. spoilage microorganism was heat treated at defined temperatures and intervals to determine its heat resistance. Except for heat treatment at 85°C, all survivor curves have regression coefficients (R^2) greater than .95 indicating strong first order kinetic models that follow linear logarithmic reductions of number of survivors over treatment time. For the first 10 minutes of heat treatment at 85°C, the spores were highly resistant and show no significant reduction in numbers - a possible slight shoulder effect that contributes to a lower regression coefficient. Additional experimentation should be completed to confirm the shoulder effect and a Weibull model may be better suited to fit the inactivation kinetics of this survival curve.

Many factors are known to effect spore heat resistance including sporulation environment, temperature and the physico-chemical properties of the heat treatment medium. An investigation on the influence of the sporulation environment temperature on the spore properties of *P. polymyxa* SQR-21, the most studied *Paenibacillus* species, demonstrated that an increase in temperature led to increased heat resistance but decreased amount of spores [14]. The incubation temperature of 30ºC for sporulation and enumeration was chosen to reflect average warehouse temperatures during the summer when beverages are in the highest demand. The presence of phosphate in the heating medium as well as an acidified medium, has been reported to lower the heat resistance of bacterial spores [15, 16]. Butterfield's buffer, a common diluent used extensively in the food industry, has a neutral pH at 7.2 but does contain phosphate in the form of KH_2PO_4. The calculated D-values at 85, 90, and 95º C are 38.3, 14.7, and 2.43 minutes,

respectively, and are similar to *P. polymyxa* spores heat treated in distilled water adjusted using HCl to pH 4. Using similar methods at 85, 90, and 95º C, their reported D values are 38.1, 10.3, and 2.7 minutes, respectively[17]. In general, the D-values and z-value determined in this study fall within the range of reported values for *Paenibacillus* spp. and *Bacillus* spp. spores [15, 16]. It may be of interest to conduct additional heat resistance studies in distilled water, as well as the acidified product.

The applied hurdles of acidification, pasteurization, and carbonation to the apple juice (10% v/v) beverage undoubtedly controlled the growth of a variety of bacteria, yeasts, and molds but not the isolated *Paenibacillus* strains. The survival curve at 95°C or 203°F, indicates that a 10 second hold time does not suffice for adequate log reductions of these spores. To control for the isolated *Paenibacillus* strains, intensity of the pasteurization hurdle should be increased with a higher temperature or longer hold time. Knowledge of the thermal inactivation parameters, the D- and z-values, for these microorganisms makes it possible to design a more effective thermal process.

CONCLUSION

Spoilage organisms of an apple juice beverage (10% v/v) withstood several designed preservation hurdles and slowly grew throughout the shelf life period, resulting in turbid bottles. The bacterial isolates have been identified as belonging to the genus *Paenibacillus*, but whole genome sequencing and traditional characterization methods should be completed to further identify the species of the spoilage isolate. The D and z values were determined for one *Paenibacillus* sp. isolate in buffer. Further work is needed to determine and compare the heat resistance of the second isolate, but considering it was isolated from the same product subjected to the same thermal process, the heat resistance is likely to be similar to that determined in this

study. This research is useful for the design of a new thermal process applied to the product with the intent of inactivating the spores and preventing future spoilage.

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