Bacillus spp. thermal resistance and validation in soups

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The purpose of this work was to obtain heat resistance data relevant to pasteurisation regimes and to model the effects of a range of temperatures (93 to 107°C) and pH (3.6 to 6.4) on the survival of the several *Bacillu*s spp. spores that have been associated with spoilage of foods *(Bacillus pumilus*, *B. licheniformis*, *B. subtilis*, and *B. megaterium)*, and to validate the results obtained by heating the spores in soups at selected temperatures. The thermal resistance observed in soups for 3 out of the 4 *Bacillus* spp. tested were higher than predicted. This may be due to the viscosity of the soup offering protection to the test organisms during the heat treatment; alternatively other ingredients in the soup recipes may cause this effect. This work will assist in the better design of thermal processing for the elimination of bacilli in soups.

Keywords *Bacillus* spp.; thermal resistance; validation

1. Introduction

Bacillus spp. are sporeforming bacteria that are widely distributed in nature, and commonly associated with a variety of food products such as milk and dairy products, meat and meat products, rice, pasta, and dried products such as spices. Spore formation allows these bacteria to survive in the environment and provides them with resistance to pasteurisation treatments. A very diverse range of pasteurised food products are now available to the consumer and with world-wide preferences for more highly spiced and flavoured foods, these products are likely to contain many different ingredients which could be contaminated with *Bacillus* species. Traditionally these microorganisms have been associated with the spoilage of food products; however, recently they have been linked to potential food poisoning issues.

1.1 Food spoilage

Bacillus pumilus, *B. licheniformis*, *B. subtilis*, and *B. megaterium* have been traditionally associated with spoilage of food products, in many cases with a pH as low as 3.9 [1]. One of the main concerns associated with these foods is that they rely on low pH to prevent and control the growth of *Clostridium botulinum*. In some instances, growth of some of these *Bacillus* species has been observed to increase pH and this may then allow the germination of any *C. botulinum* spores present in the product.

Many *Bacillus* spp. are used for the production of commercial enzymes, including proteases, lipases, and amylases among others. The production of these enzymes is usually beneficial, but in some cases it is unfortunately detrimental for the food industry. As an example, a problem that is often related to soups and products rich in carbohydrates (e.g. puddings) is referred to as "thinning". This phenomenon has been noted to occur in sterile puddings between 2 weeks and 6 months after sterilisation [2]. This is probably due to the production of saccharolytic enzymes by heat resistant *Bacillus* spp. that will break down the carbohydrates present in the product [2-5], causing a characteristic thinning and decrease in viscosity of sauces. This continues to remain a major concern for industry, but very little research has been done into this issue[2;4;6].

Thinning could happen in two different ways:

a. Spores survive the pasteurisation/commercial sterility treatment and subsequently grow, causing the characteristic spoilage.

b. Extracellular enzymes are synthesized prior to pasteurisation/commercial sterility. The process is sufficient to eliminate viable organisms but does not inactivate the preformed heat stable enzymes. These remain active after the process and cause spoilage.

One of the difficulties in detecting this type of spoilage resides in identifying the *Bacillus* species that produce the enzyme in a spoiled product. There are reports that show that some of the *Bacillus* enzymes may be resistant enough to survive a pasteurisation treatment, even heat treatments as high as UHT in puddings [2;6]. If this is the case, no bacteria would be detected when investigating a thinning spoilage. In some cases, however, it has been noted that *Bacillus* spp. have survived processes, causing spoilage by growth. A good example was reported by Thomas and Masters [4], who found that the spoilage of pre-cooked potato-topped pies was associated with amylase activity related to viable *Bacillus* spp. during storage. Additionally, there are many reports of *Bacillus* spp. surviving baking processes and causing ropiness in bread [7-10], a condition caused by the production of exopolysaccharides and the breakdown of the starches by amylases that gives bread an unpleasant and "slimy" appearance.

In some foods, the ability of a number of *Bacillus* species to produce enzymes (mostly proteases and lipases) has been used in a positive manner to produce the food. Good examples are some African and Asian fermented dishes [7;11-16]. The enzymes are used in the fermentation process of these products; for example, the Thai fermented soy

product called "Thua nao", or the African fermented locus bean product called "soumbala", and the fermented karkade called "bikalga". It is interesting to note that most of these bacilli have also been isolated from fermented sausages [17].

1.2 Epidemiology and food safety

From the food safety point of view, the most important species is *B. cereus,* known for its ability to form toxins and cause foodborne illnesses. Because of this, extensive work has been conducted on *B. cereus* heat resistance and growth characteristics. On the other hand, very little knowledge exists on the thermal resistance of the 4 *Bacillus* species in this study (Table 1).

Table 1 Results for D values reported on *B. pumilus*, *B. licheniformis*, *B. subtilis* and *B. megaterium*.

Bacteria	D value	Medium	Reference
B. pumilus	D_{104}=0.15	Distilled water	[18]
*B. pumilus**	D_{100}=0.83	UHT half-fat milk	[19]
B. megaterium	D_{100}=1.6	Distilled water	[20]
B. megaterium	D_{100}=0.8	Orange juice	[20]
B. megaterium	D_{100}=1.6	Tomato juice	[20]
B. megaterium	D_{100}=1.0	guava juice	[20]
B. megaterium	D_{100}=0.8	Mango juice	[20]
B. megaterium(3)*	D_{100}=2.16	UTH half-fat milk	[19]
B. subtilis	D_{100}=6.5	-	[21]
B. subtilis	D_{100}=5.2	Water	[22]
B. subtilis	D_{95}=5.76	Whole milk UHT	[7]
B. subtilis	D_{95}=5.13	Skimmed milk UHT	[7]
B. subtilis	D_{111}=0.2	Tryptic Soy Broth	[23]
B. subtilis (food isolate)	D_{111}=22	Tryptic Soy Broth	[23]
B. subtilis	D_{95}=9.1	0.1M PBS	[24]
B. subtilis(2) *	D_{100}=1.18	UHT half-fat milk	[19]
B. licheniformis	D_{95}=4.4	Tomato puree (pH 4.4)	[25]
B. licheniformis	D_{95}=7.8	Buffer (pH 7.2)	[25]
B. licheniformis(1) *	D_{100}=2.37	UTH half-fat milk	[19]
B. licheniformis	D_{105}=0.17	McIlvaine buffer (pH 6)	[26]

(1) value average of 21 strains
(2) value average of 18 strains
(3) value average of 3 strains
* dairy farm isolates

In addition to the spoilage cases, there is an increased concern about the potential food safety implications of these species. This remains controversial and the United States Environmental Protection Agency does not consider *B. subtilis* and *B. licheniformis* to be human pathogenic organisms [27;28]. The US Food and Drug Administration has approved the use of *B. subtilis* and *B. licheniformis* strains for commercial enzyme production [29-31]. These enzymes are divided into 3 categories and have a GRAS (Generally Recognized As Safe) status. In the description of these enzymes, the US Code of Federal Regulations (CFR), under Title 21, part 184 entitled "Direct food substances affirmed as generally recognized as safe" states:

"Sec. 184.1148 Bacterially-derived carbohydrase enzyme preparation.
Bacterially-derived carbohydrase enzyme preparation resulting from a pure culture fermentation of a **nonpathogenic and nontoxigenic strain of *Bacillus subtilis*** or *B. amyloliquefaciens*..."

"Sec. 184.1150Bacterially-derived protease enzyme preparation.
(a) Bacterially-derived protease enzyme preparation is obtained from the culture filtrate resulting from a pure culture fermentation of a **nonpathogenic and nontoxigenic strain of *Bacillus subtilis*** or *B. amyloliquefaciens*..."

"Sec. 184.1027 Mixed carbohydrase and protease enzyme product
(a) Mixed carbohydrase and protease enzyme product is an enzyme preparation that includes carbohydrase and protease activity. It is obtained from the culture filtrate resulting from a pure culture fermentation of **a nonpathogenic strain of *B. licheniformis***..."

Unfortunately, it does not specify what series of tests must be conducted to ascertain the safety of the strains. In addition, for *B. licheniformis* it is required to be nonpathogenic but does not make any reference to its potential toxigenicity.

In contrast, the Health Protection Agency (HPA) in the United Kingdom has reported a total of 17 outbreaks of gastroenteritis attributed to *B. subtilis* and 5 other outbreaks attributed to *Bacillus* spp. in England and Wales for the period 1992-2006 [32]. Previous reports by the HPA also include several cases of foodborne illnesses associated with *B. subtilis* and *B. licheniformis* [33-36]. Salkinoja-Salonen et al. [37] studied the potential toxin formation of 210 *B.*

licheniformis strains isolated or involved in foodborne poisoning cases. A total of 10 isolates were shown to produce toxin during *in-vitro* assays. From et al. [38] showed the production of pumilacidins by a *B. pumilus* strain implicated in a small foodborne outbreak related to the consumption of rice in a Chinese restaurant. The rice was shown to have a level of *B. pumilus* of 10^5cfu/g. High levels of *Bacillus* spp. may be related to foodborne cases [39], possibly by infection or by the presence of the organism causing a reaction in the consumer.

Some strains of *B. subtilis* and *B. licheniformis* have been approved for use as animal feed additives and probiotics for humans [40-44]. By contrast, some recent scientific publications raise the concern of potential toxin production from most of these species [5;37;45-48]. In many of these cases, the *Bacillus* strains used are farm or environmental isolates and are tested for toxin production or cytotoxicity *in vitro*. For example, From et al. [38] tested a total of 333 *Bacillus* spp. isolated from foods, water, and food plants and reported that only 1.8% and 0.9% of the tested species were enterotoxin or emetic toxin producers respectively, according to their *in vitro* assays.

2. Objective

The purpose of this work was to obtain heat resistance data relevant to pasteurisation regimes and to model the effects of a range of temperatures (93 to 107°C) and pH (3.6 to 6.4) on the survival of the several *Bacillu*s spp. spores that have been associated with spoilage of foods *(Bacillus pumilus*, *B. licheniformis*, *B. subtilis*, and *B. megaterium)*, and to validate the results obtained by heating the spores in soups at selected temperatures.

3. Materials and methods

3.1 Strains used and culturing conditions

Strains of *Bacillus pumilus* (NCIMB9369), *B. megaterium* (NCIMB9376), *B. licheniformis* (NCIMB9375), and *B. subtilis* (NCIMB3610) were obtained from the NCIMB (Aberdeen, Scotland). The identity of the strains was verified using a Riboprinter (Qualicon). To prepare the spore crop, each strain was inoculated into Nutrient Broth (NB, Oxoid CM1) and incubated at 37°C for 48 h. This was used to inoculate the surface of approximately 100 plates of Campden Sporulation Agar (CSA: tryptone 5g, bacteriological peptone 5g, lab lemco 1g, yeast extract 2g, $CaCl_2$ 0.056g, $MnSO_4$:$4H_2O$ 0.082g, glucose 1g, agar 15g; made up to one litre with purified water). The plates were incubated at 37°C for between 2 and 5 days, after which the growth was removed from the surface with a sterile spatula and collected in sterile distilled water. The spores were separated from cell debris by centrifuging at 2700 g for 20 min and washing twice with sterile distilled water. The spore crop was stored at refrigeration temperature until used. When needed, an appropriate volume was centrifuged, and the spores were resuspended in the heating medium (citrate-phosphate buffer) and heat-treated without delay. After heat treatment, cells were cultured by the pour plate technique using Nutrient Agar (NA, Oxoid CM3) and incubated at 37°C for 48 h.

3.2 Modelling and heat resistance determination.

The heat resistance of the spores was determined using a glass sphere technique [49]. A total of 0.1 ml of the heating medium containing spores was inserted into each glass sphere. The spheres were then heat sealed. Spheres were placed in a water-bath at the appropriate temperature and removed at different time intervals. Once the spheres had been cooled by immersion in cold water, they were placed in a 5% H_2O_2 solution to eliminate any contamination on the outside of the sphere, rinsed in sterile distilled water and placed in 10 ml MRD. The spheres were crushed with a sterile glass rod and the surviving microorganisms were enumerated using Plate Count Agar, (PCA, Oxoid CM325) The heat resistance of the spores was tested across a range of temperatures (93 to 107°C) and pH (citrate-phosphate buffer 3.6 to 6.4). Colonies were counted after incubation and the regression line was calculated between length of treatment and the decimal logarithm of the survivors. The D value (time needed to reduce the cell count 10-fold) was calculated as the reciprocal of the slope of this regression line. A surface response design was used to determine the thermal resistance across a range of temperature (93 to 107°C) and pH (3.6 to 6.4) with the central point in the composite design repeated 5 times. The results were analysed using Minitab 15 Statistical Software (Minitab Inc, PA, USA) and a predictive model for the 4 *Bacillus* species was proposed.

3.3 Validation work and heat resistance determination.

The heat resistance of the spores in the 3 different types of soups was determined using a glass capillary technique. A total of 0.05g ml of the inoculated soup containing spores was inserted into each glass capillary. The capillaries were then heat sealed. Capillaries were placed in a water-bath at the appropriate temperature (95, 100 and 105°C) and removed at different time intervals and treated as described in section 3.2. Each individual combination was repeated 3

times. Bias factor (indicates the level of agreement between predicted and observed values) and accuracy factor (how far off is the predicted value from the observed) were used to determine the performance of the model as described by Ross [50].

3.4 Soups used for the validation work.

Canned cream of tomato, potato and leek, and cream of chicken canned soups were purchased from a local supermarket. The pH of each soup was measured: cream of tomato pH=4.1, potato and leek pH=4.9, and cream of chicken pH=5.9. The ingredients in the 3 different soups were (as shown in the label):
Cream of chicken: Water, Chicken (4%), Modified Cornflour, Vegetable Oil, Cream, Dried Skimmed Milk, Wheat Flour, Flavourings, Milk Proteins, Cornflour, Yeast Extracts, Herb Extracts, Stabiliser - Polyphosphates and Sodium Phosphates, Salt, Garlic Salt, Spice Extract, Colour - Beta-carotene.
Potato and leek: Water, Potatoes (23%), Leeks (8%), Onions, Whipping Cream, Modified Cornflour, Cornflour, Salt, Black Pepper.
Cream of tomato: Tomatoes (84%), Water, Vegetable Oil, Sugar, Modified Cornflour, Salt, Dried Skimmed Milk, Milk Proteins, Cream, Spice Extracts, Herb Extract, Citric Acid.

4. Results and Discussion

The effects of temperature and pH on the heat resistance of a range of spores of *Bacillus* spp. have been investigated in this study. The results show a decrease in the thermal resistance of the 4 bacilli when the temperature was increased from 93 to 107°C and when the pH was decreased from 6.4 to 3.6. This trend was observed when the observed D values, in minutes, were plotted in relation to pH and temperature (Figure 1A). Using these values, the predictive model was then constructed to predict the behaviour of log D in relation to pH and temperature (Figure 1B).

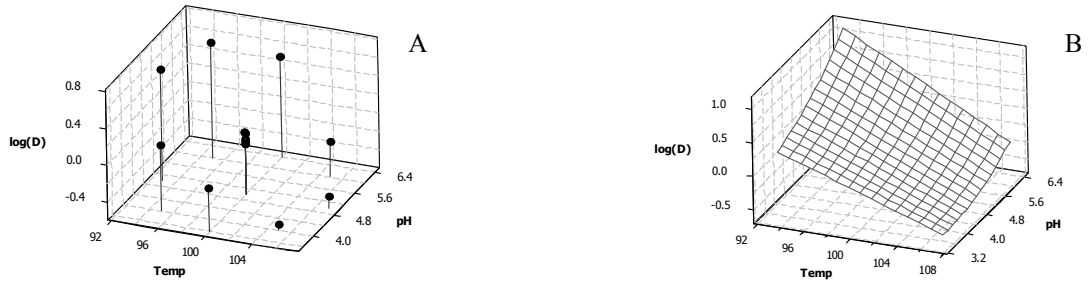

Figure 1 Example of plotting the calculated log D values (A) and the model using a response surface model (B) for *B. pumilus* thermal resistance.

The predictive models generated for the validation work used in this study can be found in Table 2.

Table 2 Predictive models for the thermal death of the spores of *B. pumilus*, *B. subtilis*, *B. megaterium* and *B. licheniformis*.

Bacillus	**Model(*)**	**R^2(%)**
B. pumilus	Log (D)= 6.541189-0.0757T + 0.215467pH	91
B. licheniformis	Log (D)= 10.17746- 0.08017T -0.99263pH +0.111101pH^2	89
B. megaterium	Log(D)= -5.52713 + 0.030831T + 3.030546pH - 0.07018pH^2 -0.02123TpH	94
B. subtilis	Log (D)= -7.74069 + 0.090036T + 2.468333pH + 0.095499pH^2 - 0.0322TpH	94

(*) Only the significant terms were included in this model

It is well known that spore heat resistance is optimum at neutral pH. The effect of reducing the pH on the thermal resistance has also been established. For example, Palop et al. [26] found that the thermal resistance of *B. licheniformis* at 99°C was 20 times higher at pH 7 when compared to pH 4. Most of the previously reported D values for *B. pumilus*, *B. megaterium*, *B. subtilis* and *B. licheniformis* have been within a narrow range of temperatures using different types of heating matrices. Montville and Sapers [25] reported a D value of 7.8 min for *B. licheniformis* at 95°C in a buffer solution with a pH of 7.2. The range of pH measured in our study is below this level; at 95°C and a pH of 6.4, the predicted D value for *B. licheniformis* was 5.75 min. A range of D values have been reported for *B. subtilis*. For

example, Kort et al. [23] reported a D value of 0.2 min at 111°C for one strain and 22 min for another *B. subtilis* strain isolated from food. For *B. subtilis,* Montville and Sapers [25] reported a D_{95} in tomato puree (pH=4.4) of 4.4 min, and a D_{95} of 7.8 min when in a buffer solution (pH=7.2). For *B. megaterium*, Gibriel and Abd-el Al [20] reported a range of D values at 100°C from 0.8 to 1.6 min in a range of fruit juices and distilled water. Ruiz et al. [18] reported a D value for *B. pumilus* of 0.15 min in distilled water at 104°C. Janstova and Lukasova [51] have also reported D values from *Bacillus* spores isolated from farm environments. The reported D values were 2.16, 2.37, and 1.18 min for *B. megaterium*, *B. licheniformis* and *B. subtilis* in diluted 1:10 UHT half milk. Variations among different species and strains have been previously reported; this may explain the differences in reported D values.

Looking at the predictive model, all the graphs show a similar pattern. Most of the previously published studies using predictive equations and surface response design have been done with *B. cereus* and *B. stearothermophilus* [52-57]. To our knowledge, there has only been one published predictive model for *B. subtilis* [7]. This was done with a pH range from 6-8, a temperature range from 89 to 98°C and the addition of sodium chloride (0 to 5%) in a potassium phosphate buffer solution.

The polynomial model used to describe the thermal resistance of bacilli in buffer was validated in different soups, tomato, potato and leek and chicken, heated at 95, 100 and 105°C. Overall bias factors of 0.56, 1.02, 0.45 and 0.46 with accuracy factors of 1.78, 1.25, 2.21 and 2.18 were observed for *B. pumilus, B. licheniformis, B. megaterium* and *B. subtilis* when heated at different temperatures for the 3 types of soup tested. Table 3 shows the bias and accuracy factors calculated for each organism and product type and temperature used for the validation studies.

Table 3 Bias and accuracy factors calculated for the validation of the model in soups.

	B. pumilus		*B. licheniformis*		*B. megaterium*		*B. subtilis*	
	Bias	Acc.	Bias	Acc.	Bias	Acc.	Bias	Acc.
Product								
Tomato	0.69	1.44	1.08	1.28	0.40	2.53	0.58	1.73
Potato	0.53	1.89	0.86	1.30	0.45	2.20	0.41	2.43
Chicken	0.48	2.08	1.14	1.19	0.52	1.93	0.41	2.45
Temp. (°C)								
95	0.57	1.25	1.37	1.37	0.40	2.47	0.44	2.29
100	0.48	2.10	1.03	1.07	0.35	2.88	0.39	2.44
105	0.65	1.55	0.75	1.34	0.66	1.51	0.27	1.85

Jagnannath et al. [7] validated the results of their model for the thermal resistance of *B. subtilis* spores in milk. The authors found that the predicted D values for *B. subtilis* spores in buffers were higher than those found in milk, concluding that the predictions were fail-safe. In our study, the predicted model for *B. licheniformis* was fail-safe, whereas those for the other 3 bacilli were fail-dangerous (Figure 2).

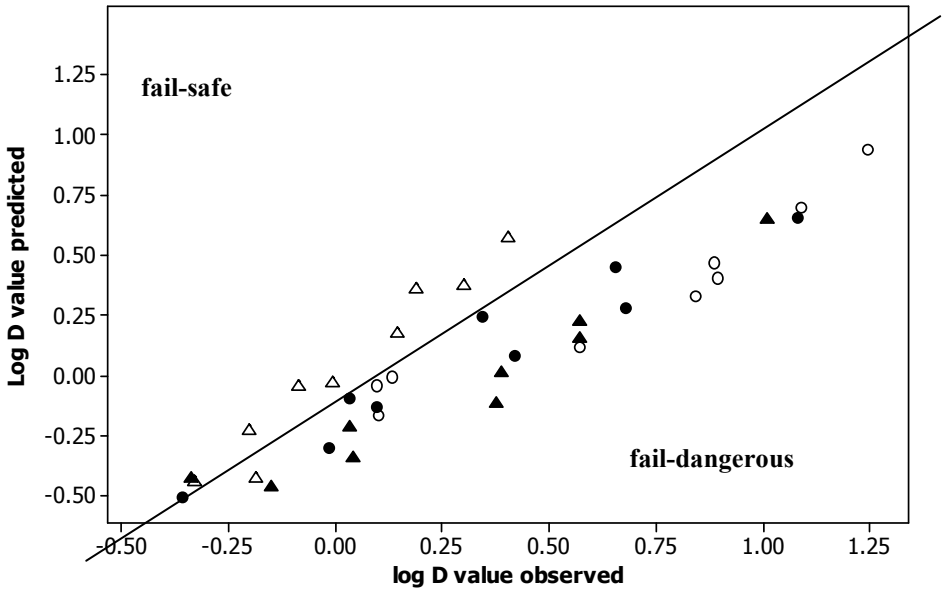

Figure 2. Graphical comparison of predicted vs. observed values for the thermal resistance of the spores of *B. pumilus* (●), *B. licheniformis* (△), *B. megaterium* (○) and *B. subtilis* (▲) in soups.

As opposed to the study by Jagnannath et al. [7] in which only one *Bacillus* spp. was used, in our study, 4 different bacilli were used. Despite the fact that we have seen that the model can be fail-safe for 1 species, the model failed to predict the thermal resistance (fail-dangerous) for the other 3 bacilli.

5. Concluding remarks

This is the first study to report the heat resistance of these 4 *Bacillus* species across a range of pasteurisation temperatures and pHs. The initial study using buffers adjusted to pH 3.6 to 6.4 enabled us to conclude that reducing the pH also reduces the heat resistance of these bacilli; this has a larger effect when processing at lower temperatures. The thermal resistances observed in soups for 3 out of the 4 *Bacillus* spp. tested were higher than predicted. This may be due to the viscosity of the soup offering protection to the test organisms during the heat treatment; alternatively other ingredients in the soup recipes may cause this effect. This work will assist in the better design of thermal processing for the elimination of bacilli in soups.

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