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Impact of temperature, nutrients, pH and cold storage on the germination, growth and resistance of *Bacillus cereus* spores in egg white

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ABSTRACT

B. cereus spores are a concern to the food industry, especially to the producers of heat sensitive food products like egg white or precooked and stored food such as fried rice. This study investigated the impact of nutrients, temperature (4, 8, 15 and 25 °C), pH (4, 5, 7 and 9), and cold storage on the germination, growth and resistance of *B. cereus* spores. In egg white held at 4 °C for 12 days spore germination was not apparent, however the addition of egg yolk (5%) resulted in a 2 Log colony forming units (CFU)/mL increase in vegetative cells ($p < .05$). Adding L-alanine (0.9 mg/mL) to egg white did not induce germination unless the spores were simultaneously heat activated at 70 °C for 30 min. On incubation at 15 or 25 °C in egg white, spore germination increased by 3.0 Log and 3.7 Log CFU/mL on day 12. The presence of 5% yolk further enhanced germination and subsequent sporulation during storage at 15 and 25 °C. Acidification (pH 4) of 10% egg white solution prevented germination at 4, 8, 15 and 25 °C. Spores held at 4 °C for 6 days in phosphate buffer (50 mM, pH 4) had visible deformations on their surface (scanning electron microscopy) and a significant reduction in $D_{88,}$ and D_{92} values of 13.9 and 8.2 min respectively. A better understanding of how spores sense and respond to changing environmental conditions will help in the development of processing strategies, involving multiple hurdles to ensure the prevention of germination and subsequent toxin production.

1. Introduction

Egg white is a rich source of proteins, especially amino acids such as isoleucine (50.2 mg/mL), leucine (71.2 mg/mL) and glutamic acid (114.9 mg/mL) (Ruxton, Derbyshire, & Gibson, 2010). Microorganisms such as *Bacillus cereus* can contaminate egg white either during its development in the oviduct, via on farm handling or during processing. *B. cereus* is a rod shaped spore forming aerobic or facultative anaerobic bacteria capable of causing food poisoning (Andersson, Ronner, & Granum, 1995; Ceuppens et al., 2011; Daelman et al., 2013). As *B. cereus* spores are not killed by the heat regimes of 60 °C for 3 min (Cunningham, 1995) designed to pasteurise liquid egg products, these spores have been found in pasteurised whole egg, egg white and yolk (Gast, Guraya, Guard-Bouldin, & Holt, 2007; Postollec, Mathot, Benard, & ML, D., Pavan, S., & Sohier, D., 2012; Rivoal et al., 2010). If conditions become favourable, the germination of *B. cereus* spores in foods and the subsequent growth of the vegetative cells can lead to the production of an emetic (cereulide) toxin, with the number of cells reported to produce sufficient toxin to cause illness ranging from 10^3 to 10^5 cells/g (Kramer & Gilbert, 1989; Rajkovic et al., 2006. The emetic toxin (cereulide) has been associated with specific strains that are more commonly found in heat-processed foods or preheated foods that are kept warm (Carlin et al., 2006; Stark et al., 2013). The production of emetic toxin is also highly influenced by the food matrix, incubation conditions (aeration or shaking) and temperature of incubation (Rajkovic et al., 2006). The emetic toxin has been reported as being the main cause of death of a 20-year old individual who consumed pasta infected with *B. cereus* (Naranjo et al., 2011). If food containing vegetative cells (infective doses 10^4–10^9 cells/g of food) is ingested, diarrheal toxins (Nhe, Hbl and Cyt K) can subsequently be produced in the small intestine (Granum & Lund, 1997). The non-haemolytic enterotoxin (Nhe) has been associated with a food poisoning outbreak in Norway in 1995 (Lund & Granum, 1996) while cytotoxin K has been associated with a food poisoning outbreak in France, that resulted in 3 deaths (Lund, De Buyser, & Granum, 2000). These and other food poisoning outbreaks attributed to *B. cereus* have highlighted the need for better control measures. As the prevention of germination will eliminate the threat of toxin production by the vegetative cells, a better understanding of the factors influencing spore germination is required.

Germination is a complex two-stage process. The first stage involves the release of ions and dipicolinic acid (DPA), the partial rehydration of the core and a slight loss of heat resistance (Setlow, 2003). The second

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stage consists of cortex hydrolysis, core rehydration and a further loss of heat resistance, followed by outgrowth. A number of methods have been used to monitor bacterial spore germination including, optical density measurements, DPA release and live cell imaging (Pandey et al., 2013; Yi & Setlow, 2010). However, these methods are not 100% reliable in assessing germination as core rehydration and cortex lysis may result in the onset of germination but not complete outgrowth into vegetative cells, also some strains lack DPA (Wise, Swanson, & Halvorson, 1967) and live cell imaging cannot take the whole heterogeneous population into account. Another important factor to be considered is that *B. cereus* strains show a wide variation in nutritional requirements, physiological and metabolic characteristics, heat stability and germination rates (Abee et al., 2011; Lawley, Curtis, & Davis, 2012).

In the current study, a modified method reported by Seydlova and Svobodova (2012) was used to allow the time-dependent monitoring of spore germination. Total microbial number (TMN) and spore number (SN) were assessed by plating the dilutions onto plate count agar (PCA) plates before and after they were subjected to a heat treatment at 80 °C for 15 min. In general, the TMN encompasses both vegetative cells and spores, while SN obtained after heat treatment relates only to spores that retain heat resistance. The difference between TMN and SN is the number of vegetative cells or heat sensitive spores killed by the mild heat treatment. If TMN and SN are the same, this indicates that only heat resistant spores are present. A decrease in SN with no change in TMN indicates that the spores are germinating, while an increase in TMN indicates that the recently germinated cells are reproducing. Though the plating method is more time consuming than optical density measurements, it is a precise and cost effective way to enumerate the dormant (completely resistant) and germinating cells. The onset of germination or incomplete germination, which is not possible to determine by plating alone can be monitored by determining the decimal reduction time (D-values) of the spores (Awuah, Ramaswamy, & Economides, 2007).

The objectives of this study were to firstly investigate germination of *B. cereus* spores in egg white held under conditions usually encountered during processing and storage and secondly to determine the effect of nutrients (egg yolk or L-alanine), temperature, pH, heat activation and cold storage on the germination, growth and resistance of *B. cereus* spores.

2. Materials and methods

2.1. Isolation and identification of the B. cereus strain used in this study

The B. cereus strain (NZAS01) used in this study was isolated from liquid egg white using an enrichment technique. Egg white (1 mL) was added to tryptic soy broth (TSB, 9 mL) and incubated at 25 °C for 48 h. The resulting culture was diluted (1:10) with peptone water (0.1%) and held at 80 °C for 15 min before being spread onto PCA plates, which were incubated at 30 °C for 48 h. This isolation method resulted in a single colonial type being visible, representative samples of which were streaked for purity onto PCA plates. The purified isolates were subsequently maintained on Luria (LB) agar plates and stored at −80 °C in 20% glycerol. Isolates were tentatively identified as *B. cereus* based on their growth and appearance on *B. cereus* selective media (*B. cereus* agar base-7442 with Polymyxin B 50,000 IU, Acumedia). A representative strain was subsequently identified using high throughput Illumina sequencing by NZGL (New Zealand Genomics Ltd., http://www.nzgenomics.co.nz) at the University of Otago (Dunedin, New Zealand). For DNA isolation, the isolate was grown on Luria agar for 24 h at 37 °C. DNA was isolated using the Mo Bio™ Microbial DNA Isolation Kit (GeneWorks NZ, Ltd.) following the manufacturer's instructions. Illumina sequencing libraries were prepared using the Illumina TruSeq DNA sample preparation V2 kit following the standard low throughput protocols in which, one microgram of each genomic DNA was sheared to approximately 350 bp size using the Covaris S2 (Covaris, Woburn, MA, USA). End repair, A-tailing and addition of barcoded adaptors were performed as per the standard low throughput protocol. Ligation products were run on a 2% 1 × TAE gel and bands of 400–500 bp were excised, purified and enriched by PCR amplification following Illumina's protocols. The isolate was sequenced to 300 bp on a MiSeq system and demultiplexed using the Illumina Casava application, v 1.8.2. The quality of the raw reads was checked using FastQC program (Andrews, 2015). The Illumina adaptor sequences were trimmed from the reads using clean adaptors (Chatterjee, Stockwell, Rodger, & Morison, 2012).

2.2. Preparation of spore suspension

An inoculum from the *B. cereus* stock culture stored at −80 °C was streaked onto a PCA plate, which was incubated at 30 °C for 48 h. A single colony was subsequently inoculated into a 100 mL of TSB and incubated at 30 °C for 72 h in an orbital shaker incubator at 75 rpm. The resulting culture was harvested by centrifugation (15,300 *g* for 8 min at 4 °C) and washed three times with autoclaved distilled water. The crude spore suspension was suspended in distilled water and stored at −18 °C.

2.3. Estimation of total microbial number (TMN) and spore number (SN)

The total microbial number (TMN) in the resulting suspension was determined by making serial dilutions in 0.1% peptone solution and plating them onto PCA plates (in triplicates). The spore numbers (SN) in the suspension were determined by the method used by Kim et al. (2014) which involved heating the suspension (1 mL) in a closed sterile Eppendorf tube (volume 1.5 mL) at 80 °C for 15 min in a water bath and immediately transferring the tube to ice slurry, before diluting and plating as outlined above. The plates were incubated at 30 °C and the number of colonies counted after 48 h of incubation and cross-checked after 72 h to rule out the possibility of slow growth. TMN represent the number of colony forming units derived from both the vegetative cells and spores present while the SN represents the number of colonies derived from spores alone. The difference between TMN and SN represents the number of vegetative cells and partially germinated spores (heat sensitive) present in the suspension and was used as a proxy to estimate germination. Counts were presented as CFU/mL.

2.4. Quantification of dipicolinic acid (DPA)

The release of DPA during the germination of *B. cereus* spores into the suspension/media was estimated using the method described by Scott and Ellar (1978). The supernatant from samples (1 mL) at all time points was collected by centrifugation (15,300 *g* for 5 min). The maximum absorption was found to be at 270 nm for the supernatant using a wave scan (200 nm–800 nm range) which was similar to Ca-DPA and DPA secreted by *B. cereus* during germination (Bailey, Karp, & Sacks, 1965; Scott & Ellar, 1978). A standard curve of DPA (Sigma Aldrich) ranging from 0 to 200 μg/mL was used to determine the sample concentrations.

2.5. Challenge testing using nutrients — L-alanine and egg yolk

To study the effect of either egg yolk or L-alanine on spore germination, egg white (10 mL) alone or egg white containing either 1% or 5% (*w/v*, total mixture volume of 10 mL) egg yolk or L-alanine (0.9 mg/mL) was inoculated with *B. cereus* spores to achieve a final number of 10^4 CFU/mL. All trials were carried out on three replicate samples. The egg white and yolk used was obtained from fresh eggs (less than a week old), after their surfaces were disinfected using 70% ethanol, prior to the yolk being aseptically separated from the egg white. Egg white was checked for any initial microbial contamination before inoculation. The