

Optimal Growth Conditions for Testing UHT Oat Milk for the Presence of *Bacillus* and *Geobacillus* species using the Innovate System

Objective

Many food manufacturers still use traditional plating methods to detect contamination in finished product. However, this process can take 7 - 15 days, requiring significant technician time for plating and counting colonies. To reduce the time to results, reduce costs, and increase efficiency, the Innovate System can be used to generate results in as little as 24 - 48 hours, depending on the organism, level of contamination, and the matrix being evaluated. In this study, we examined the growth characteristics of gram-positive spore formers, *Bacillus* and *Geobacillus*, in UHT oat milk to determine the best parameters for detection as a low-level contaminant.

Methods and Reagents

Summary

Commercially available oat milk was purchased and used to support the growth of the following organisms: *Bacillus cereus*, *B. licheniformis*, *B. thuringiensis* and *Geobacillus stearothermophilus*. A total of 60 packs were used for the study, using oat milk to serially dilute each organism.

The organisms were grown over night in tryptic soy broth (TSB) at 33 °C to stationary phase and directly spiked into 110 mL aliquots of milk, this was then serially diluted to extinction (-10). Then, 100 mL of each dilution was sterile-injected into each pack following sterile removal of 100 mL of internal milk.

The inoculated packs were incubated at 25 °C, 33 °C or 55 °C and assayed in triplicate at 0, 4, 8, 24, and 48 hours using the Innovate system and the RapiScreen™ Dairy Kit and protocol.

Identification of known and unknown samples

The identifications of all known and unknown isolates were verified using the RiboPrinter®, following the established protocol. In addition, five independent colonies were used to verify each isolate using *ECORII* typing.

Results

Growth of *Bacillus cereus*

At each time point and for each serial dilution of *B. cereus* culture, spiked oat milk cartons were analyzed for organism growth. Results are shown in the graphs and tables below. Results demonstrate that the growth of *B. cereus* at all temperatures is very rapid. At 25 °C, stationary phase is reached within 24 hours (Figure 1), the ATP level being around 100,000 RLU (Table 1). At 33 °C a similar stationary phase of 100,000 RLU is reached but in a shorter timeframe (approx. 12 hours; see Figure 2 and Table 2). This increase in incubation temperature appears to select for quicker growth of the organism. At 55 °C, a similar growth rate is seen (when compared to 33 °C) but a higher stationary RLU seems to be reached around 1,000,000 RLU (Figure 3, Table 3). Collectively, this data shows that *B. cereus* is a very adaptable, rapidly growing, and could easily cross-contaminate clean surfaces or liquids if left unchecked.

Figure 1. Growth Curve at 25 °C

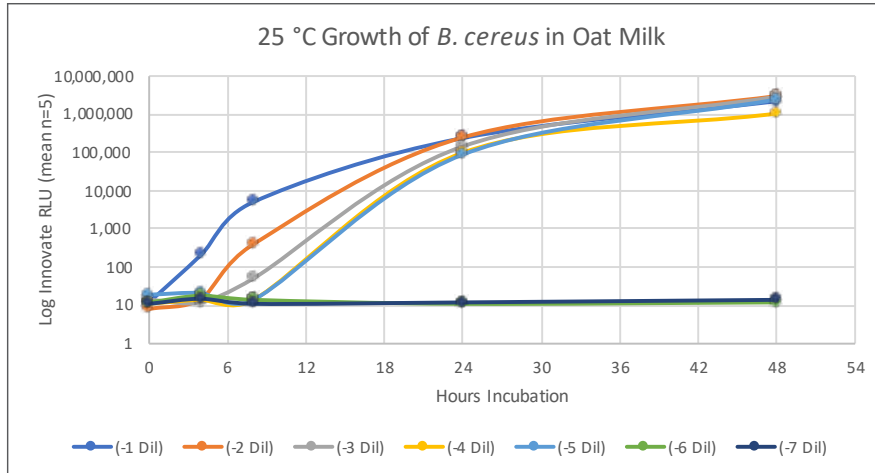


Table 1: RLU Values at 25 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 12 | 8 | 14 | 11 | 19 | 12 | 11 |
| 4 | 214 | 15 | 12 | 14 | 21 | 18 | 15 |
| 8 | 5,114 | 402 | 51 | 14 | 14 | 14 | 11 |
| 24 | 238,847 | 248,814 | 144,518 | 98,801 | 88,471 | 11 | 12 |
| 48 | 2,188,474 | 3,001,547 | 2,884,744 | 1,045,099 | 2,441,744 | 12 | 14 |

Figure 2. Growth Curve at 33 °C

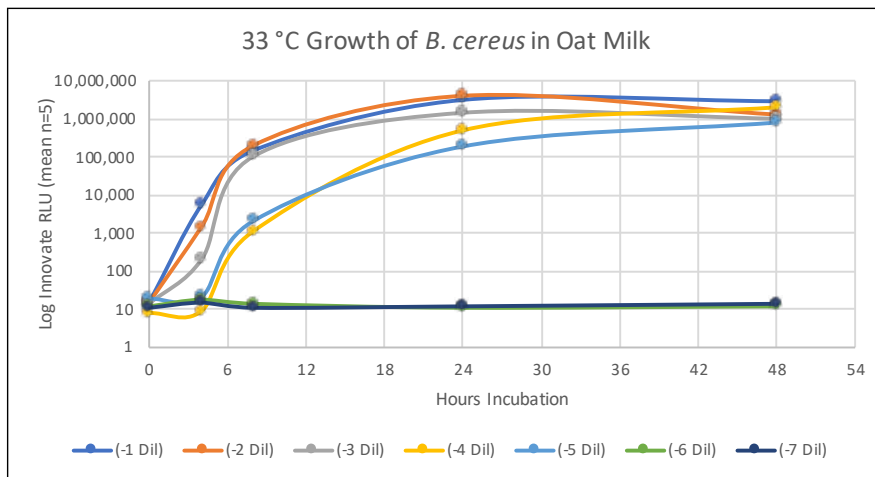


Table 2: RLU Values at 33 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 15 | 15 | 14 | 8 | 19 | 12 | 11 |
| 4 | 5,441 | 1,447 | 201 | 9 | 21 | 18 | 15 |
| 8 | 148,124 | 201,481 | 104,471 | 1,144 | 2,114 | 14 | 11 |
| 24 | 3,141,415 | 4,022,145 | 1,442,511 | 498,801 | 188,471 | 11 | 12 |
| 48 | 2,889,741 | 1,288,741 | 1,002,514 | 2,011,441 | 800,141 | 12 | 14 |

Figure 3. Growth Curve at 55 °C

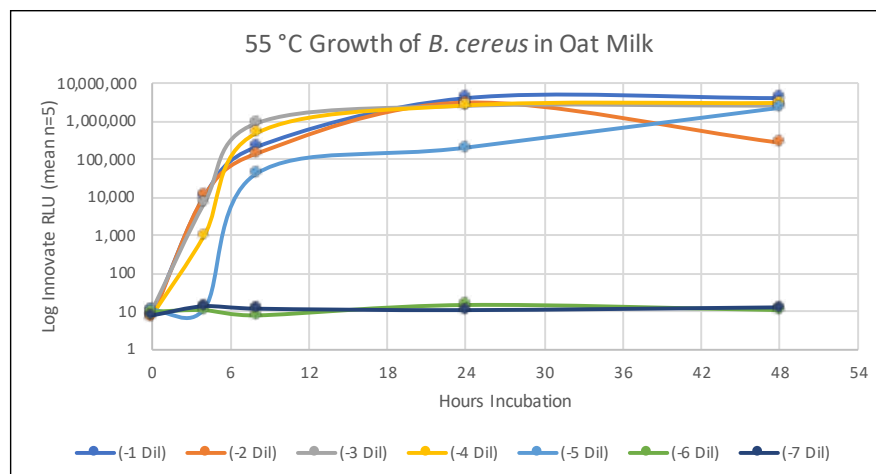


Table 3: RLU Values at 55 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 8 | 7 | 11 | 8 | 11 | 10 | 8 |
| 4 | 10,801 | 11,019 | 7,141 | 1,021 | 12 | 11 | 14 |
| 8 | 211,415 | 141,481 | 884,192 | 488,412 | 42,251 | 8 | 12 |
| 24 | 4,012,514 | 3,081,417 | 2,518,140 | 2,588,941 | 201,774 | 15 | 11 |
| 48 | 4,021,151 | 271,415 | 2,533,694 | 3,001,941 | 2,236,558 | 11 | 13 |

Growth of *Bacillus licheniformis*

At each time point and for each serial dilution of *B. cereus* culture, spiked oat milk cartons were analyzed for organism growth. Results are shown in the graphs and tables below. The growth of *B. licheniformis* is zero at 25 °C; this organism does not grow at this psychrophilic temperature (Figure 4, Table 4). At 33 °C, the organism does grow but growth is sluggish and does not reach stationary phase during the 48 hours measured (Figure 5). The RLU produced at 24 hours, between 10,000 and 100,000 are still significant and continue to increase to 1,000,000 at 48 hours (Table 5). At 55 °C, this organism grows very rapidly and reaches stationary phase in under 24 hours, indicating that this organism is thermophilic in origin and poses the risk of spores surviving heat treatment.

Figure 4. Growth Curve at 25 °C

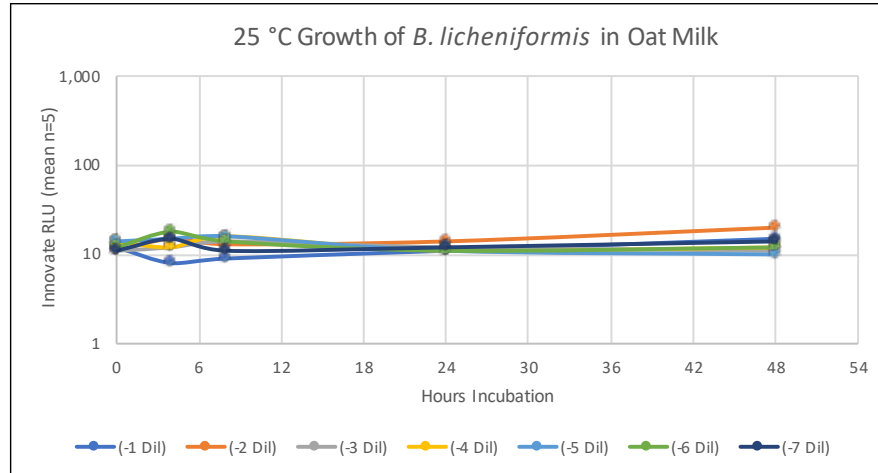


Table 4: RLU Values at 25 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 0 | 12 | 11 | 11 | 14 | 14 | 12 | 11 |
| 4 | 8 | 15 | 12 | 12 | 15 | 18 | 15 |
| 8 | 9 | 13 | 14 | 16 | 16 | 14 | 11 |
| 24 | 11 | 14 | 12 | 11 | 11 | 11 | 12 |
| 48 | 15 | 20 | 11 | 12 | 10 | 12 | 14 |

Figure 5. Growth Curve at 33 °C

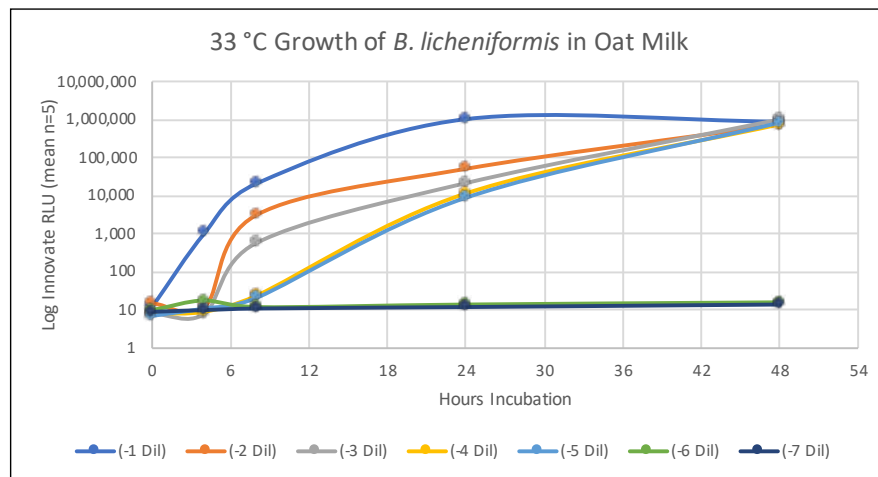


Table 5: RLU Values at 33 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 0 | 12 | 15 | 8 | 8 | 7 | 10 | 9 |
| 4 | 1,025 | 12 | 8 | 9 | 11 | 18 | 10 |
| 8 | 20,984 | 3,144 | 581 | 24 | 21 | 12 | 11 |
| 24 | 1,024,477 | 50,874 | 21,484 | 11,484 | 8,874 | 14 | 12 |
| 48 | 877,422 | 744,874 | 1,002,541 | 750,147 | 829,987 | 16 | 14 |

Figure 6. Growth Curve at 55 °C

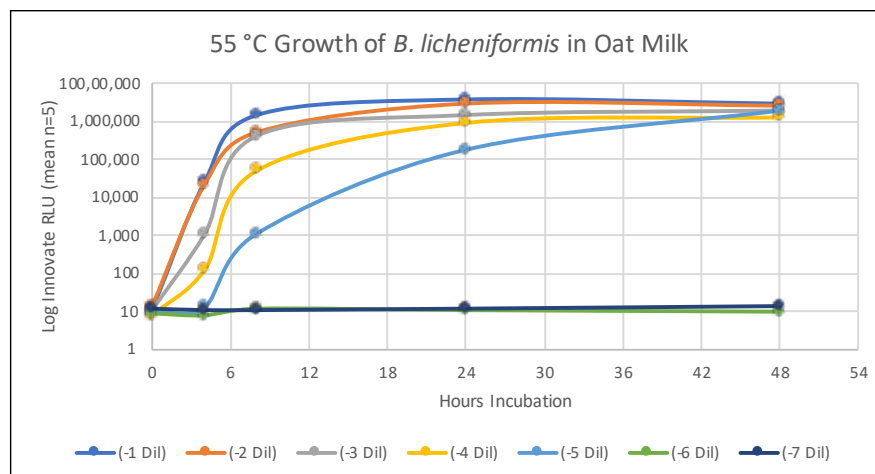


Table 6: RLU Values at 55 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 11 | 14 | 11 | 8 | 10 | 9 | 12 |
| 4 | 25,014 | 21,441 | 1,055 | 122 | 13 | 8 | 11 |
| 8 | 1,445,126 | 509,544 | 411,847 | 50,144 | 1,110 | 12 | 11 |
| 24 | 3,715,844 | 2,887,696 | 1,448,750 | 899,471 | 177,487 | 11 | 12 |
| 48 | 2,877,901 | 2,549,912 | 1,902,364 | 1,255,874 | 1,887,487 | 10 | 14 |

Growth of *Geobacillus stearothermophilus*

At each time point and for each serial dilution of *G.stearothermophilus* culture, spiked oat milk cartons were analyzed for organism growth. Results are shown in the graphs and tables below. The growth of *G. stearothermophilus* was zero at 25 and 33 °C, confirming that this organism does not grow at these psychrophilic or mesophilic temperatures (Figures 7 and 8, Tables 7 and 8). At 55 °C, the organism grows rapidly and reaches stationary phase in under 24 hours (Figure 9). The RLUs produced at 24 hours range from 200,000 to 1000,000 and remain static until 48 hours (Table 9). This demonstrates that the organism is a strict thermophile and poses the risk of spores surviving heat treatment.

Figure 7. Growth Curve at 25 °C

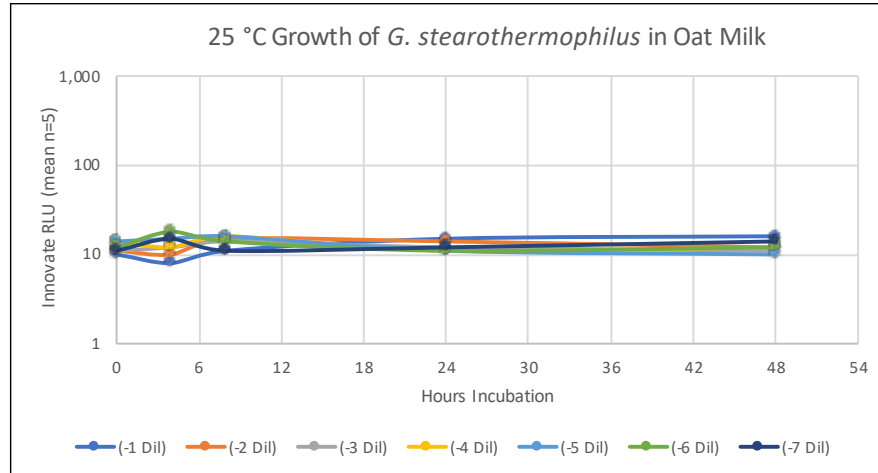


Table 7: RLU Values at 25 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 0 | 10 | 11 | 11 | 14 | 14 | 12 | 11 |
| 4 | 8 | 10 | 12 | 12 | 15 | 18 | 15 |
| 8 | 11 | 15 | 14 | 16 | 16 | 14 | 11 |
| 24 | 15 | 14 | 12 | 11 | 11 | 11 | 12 |
| 48 | 16 | 12 | 11 | 12 | 10 | 12 | 14 |

Figure 8. Growth Curve at 33 °C

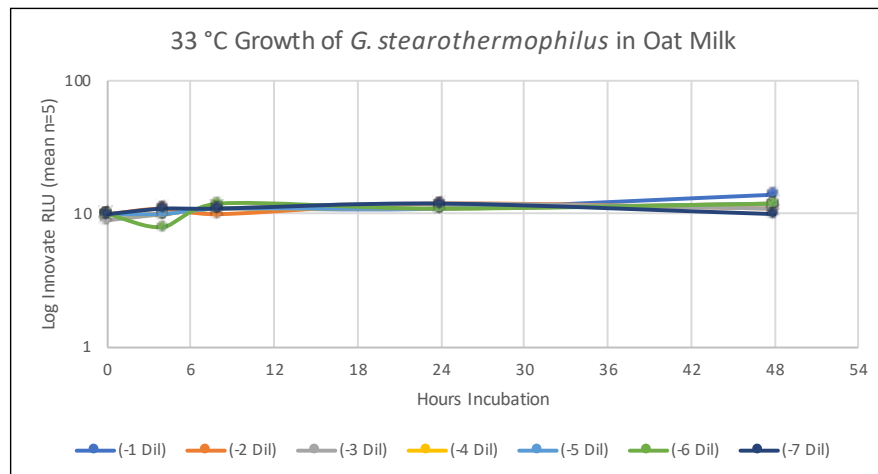


Table 8: RLU Values at 33 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 0 | 10 | 10 | 9 | 10 | 10 | 10 | 10 |
| 4 | 10 | 11 | 10 | 10 | 10 | 8 | 11 |
| 8 | 11 | 10 | 11 | 11 | 11 | 12 | 11 |
| 24 | 11 | 12 | 12 | 11 | 11 | 11 | 12 |
| 48 | 14 | 11 | 11 | 12 | 12 | 12 | 10 |

Figure 9. Growth Curve at 55 °C

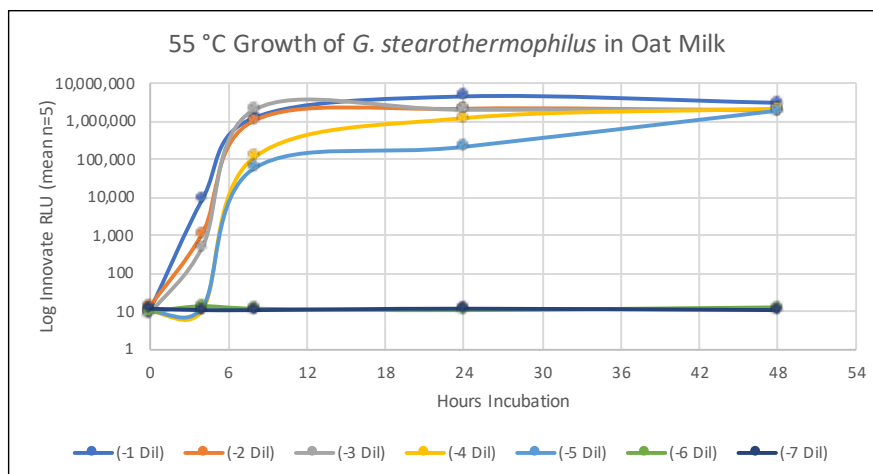


Table 9: RLU Values at 55 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 11 | 14 | 9 | 10 | 10 | 10 | 12 |
| 4 | 8,944 | 1,141 | 500 | 11 | 13 | 14 | 11 |
| 8 | 1,244,584 | 1,054,411 | 2,014,991 | 114,994 | 58,847 | 12 | 11 |
| 24 | 4,450,122 | 2,114,181 | 1,984,471 | 1,200,141 | 211,410 | 11 | 12 |
| 48 | 3,025,548 | 1,894,122 | 2,015,141 | 2,114,181 | 1,887,487 | 13 | 11 |

Growth of *Bacillus thuringiensis*

At each time point and for each serial dilution of *B. thuringiensis* culture, spiked oat milk cartons were analyzed for organism growth. Results are shown in the graphs and tables below. The growth of *B. thuringiensis* is rapid at 25 °C, with peak growth at 24 - 48 hours (Figure 10, Table 10). At 33 °C, similar growth is seen but with higher RLU values (Figure 11, Table 11), suggesting more rapid growth. Growth appears to slow at 24 - 48 hours. At 55 °C, the organism does not grow, indicating that it cannot withstand thermophilic environments (Figure 12, Table 12).

Figure 10. Growth Curve at 25 °C

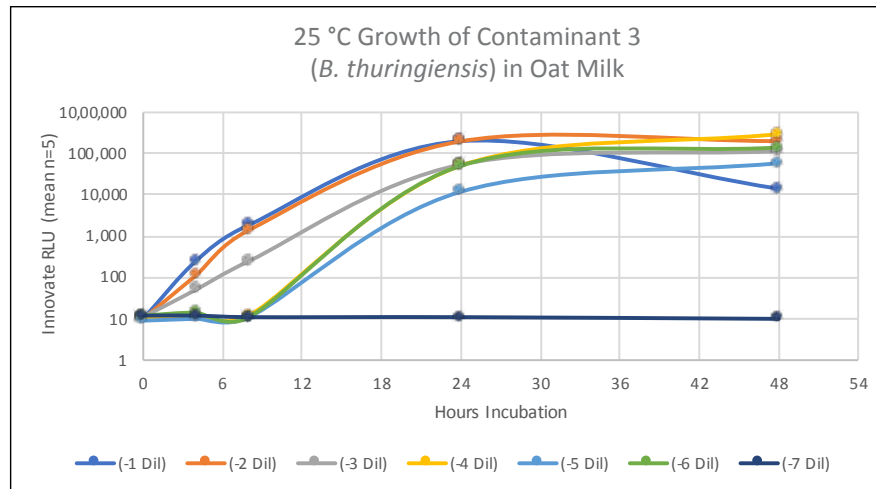


Table 10: RLU Values at 25 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 10 | 10 | 11 | 10 | 9 | 12 | 12 |
| 4 | 254 | 114 | 51 | 12 | 10 | 14 | 12 |
| 8 | 1,884 | 1,415 | 251 | 12 | 11 | 11 | 11 |
| 24 | 201,445 | 199,841 | 54,712 | 51,488 | 12,005 | 51,014 | 11 |
| 48 | 14,155 | 201,448 | 114,811 | 298,447 | 58,874 | 141,411 | 10 |

Figure 11. Growth Curve at 33 °C

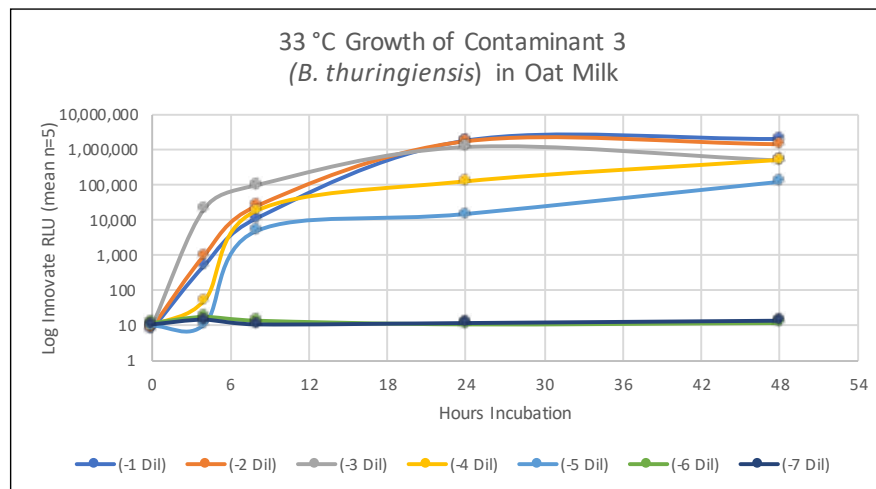


Table 11: RLU Values at 33 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| 0 | 8 | 9 | 10 | 10 | 10 | 12 | 11 |
| 4 | 523 | 1,021 | 21,441 | 53 | 11 | 18 | 15 |
| 8 | 11,451 | 25,514 | 100,211 | 18,877 | 5,001 | 14 | 11 |
| 24 | 1,844,755 | 1,766,584 | 1,211,433 | 128,411 | 15,141 | 11 | 12 |
| 48 | 2,051,600 | 1,455,011 | 501,214 | 522,641 | 124,184 | 12 | 14 |

Figure 12. Growth Curve at 55 °C

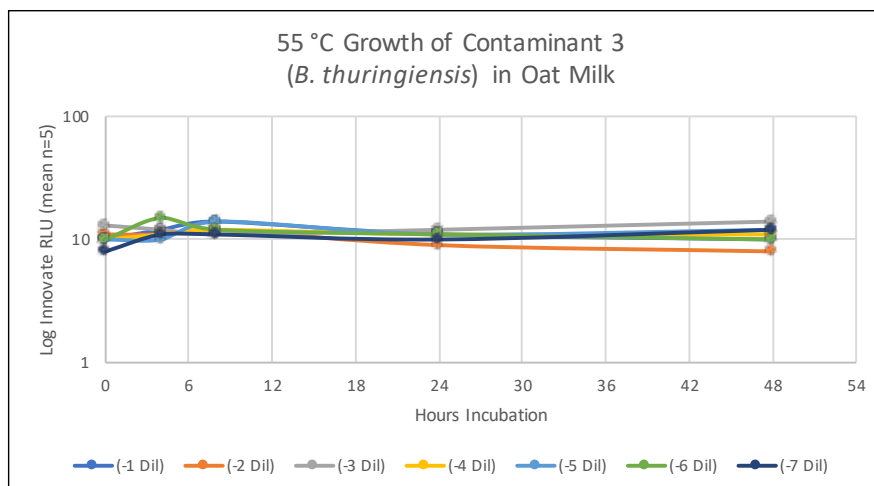


Table 12: RLU Values at 55 °C

| Hours | 10 ⁻¹ Dilution | 10 ⁻² Dilution | 10 ⁻³ Dilution | 10 ⁻⁴ Dilution | 10 ⁻⁵ Dilution | 10 ⁻⁶ Dilution | 10 ⁻⁷ Dilution |
|-------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 0 | 12 | 9 | 12 | 14 | 12 | 10 | 8 |
| 4 | 21 | 15 | 11 | 15 | 11 | 15 | 11 |
| 8 | 20 | 11 | 12 | 12 | 15 | 12 | 11 |
| 24 | 19 | 18 | 11 | 11 | 17 | 11 | 10 |
| 48 | 15 | 21 | 13 | 10 | 13 | 10 | 12 |

Data Summary

Based on the results of this study, recommendations can be made for incubation time and temperature for detection of *Bacillus* and *Geobacillus* species using the Innovate System with the RapiScreen Dairy Kit. These are summarized in the table below (Table 13). However, it is recommended that any facility validate this process for their specific matrix and relevant organism strains/species.

Table 13: Data Summary

| Organism | 25 °C | 33 °C | 55 °C |
|---------------------------------------|--------------|--------------|--------------|
| <i>Bacillus cereus</i> | 12 hours | 8 hours | 8 hours |
| <i>Bacillus licheniformis</i> | Not detected | 18 hours | 12 hours |
| <i>Geobacillus stearothermophilus</i> | Not detected | Not detected | 8 hours |
| <i>Bacillus thuringiensis</i> | 18 hours | 12 hours | Not detected |

Conclusion

Summary of Results

As a result of these studies, general conclusions can be made regarding the growth of these specific organisms in UHT oat milk.

| Hours | 25 °C | 33 °C | 55 °C |
|-------|---|--|---|
| 6 | Not detected | Not detected | <i>B. cereus</i> , <i>B. licheniformis</i> , <i>G. stearothermophilus</i> |
| 8 | Not detected | <i>B. cereus</i> , <i>B. licheniformis</i> , <i>B. thuringiensis</i> | <i>B. cereus</i> , <i>B. licheniformis</i> , <i>G. stearothermophilus</i> |
| 12 | <i>B. cereus</i> , <i>B. thuringiensis</i> | <i>B. cereus</i> , <i>B. licheniformis</i> , <i>B. thuringiensis</i> | <i>B. cereus</i> , <i>B. licheniformis</i> , <i>G. stearothermophilus</i> |
| 18 | <i>B. cereus</i> , <i>B. thuringiensis</i> | <i>B. cereus</i> , <i>B. licheniformis</i> , <i>B. thuringiensis</i> | Not detected |

Overall

Oat milk will support the growth of gram-positive spore forming organisms of the *Bacillus* and *Geobacillus* species. However, the time to detection will depend on the specific organism being grown as well as the incubation temperature. As a result, to identify all possible contaminants in oat milk, it is advisable to test a range of temperatures and incubation times – as shown here, evaluations were done from 25 °C to 55 °C and from 8 hours to 24 hours. In addition, if there is a risk of thermophilic spore formers, then samples should be incubated at 55 °C before testing. Otherwise, incubation at 33 °C should be sufficient.

No matter the concern, when spore-forming bacteria are a risk, analysis of finished product should be assessed using two variables. The first, time to results, should be based around the highest probability of detection both low and high CFU levels. Therefore, the incubation time to detections should be kept as short as possible (24 - 48 hours here) while still allowing for the detection of other organisms that may grow more slowly but still are able to be detected in the same time frame. The second variable, the growth and senescence of gram-positive spore formers, should prevent extension of the incubation time into the period where the organisms are sporulating, introducing a risk of non-detection as ATP levels fall off. Collectively, this means optimizing the two, limiting incubation to 72 hours for spore-formers while maximizing the time for growth of other slow-growing organisms.