Investigating Biofilm Formation, Enzyme Activity and Sporulation in Varied Total Dissolved Solids of *Bacillus licheniformis*

Ziyang Liu

August 2024

Abstract

This study investigated the sporulation, biofilm formation and enzymatic activity of isolates of *Bacillus licheniformis* sourced from dairy environments including butter, milk protein concentrate (MPC) and milk treatment skim module (MTSM) and from farm.

Sporulation analysis with a two way ANOVA has shown significant effects of both time and TDS level on sporulation across all samples ($p < 0.05$). All isolates tend to show higher spore counts(especially for isolates sourced from factory) with increased TDS level. This suggests potential disruption in metabolic pathways with increased ion concentrations and prompting sporulation, even in nutrient rich environments.

Biofilm was assessed at temperatures ranging from 20℃ to 60℃ using crystal violet assay. One way ANOVA indicated the temperature has a significant ($p < 0.05$) effect on biofilm forming for all isolates. Most isolates showed an optimal growth temperature between 30℃ to 50℃ with minimal growth at 20℃ and 60℃. Butter isolates peaked at 30℃ and 37℃, MPC isolates peaked at 50℃ which reflected potential adaptation to specific processing environments from their source.

Enzymatic activity screening has shown protease activity at 37℃ after 40 hours incubation and 30℃ after 72 hours incubation. Heat sensitivity tests showed no detectable result suggesting that the enzymes were either not released to the media or concentrations were too low for detection.

The findings highlight the variety of adaptive mechanisms of *Bacillus licheniformis* to environmental factors. Understanding these responses is essential for developing strategies to control the bacterial contamination in food products and processing. Future research is recommended to use more sensitive detection methods such as flow cytometry for spore counts,viable cell enumeration method (CFU/ml assay) for biofilm and azocasein assay for protease activity to improve accuracy.

Table of Contents

1.0 Introduction

Microbial management is important for ensuring food quality and longevity. Spoilage organisms can be introduced to food products via a variety of ways such as environment, handling and processing (Alegbeleye et al., 2022). *Bacillus licheniformis* is a spore forming bacterium and known for its extensive application in the food industry. It has various enzyme activities and the ability to survive in harsh processing environments. These characteristics give *Bacillus licheniformis* great potential for producing microbial compounds in diverse environments. However, while this microbe enhances the production of a wide range of bioproducts, it also presents challenges by causing food spoilage especially in the dairy industry (Wang et al., 2021). The dual role highlights the need for effective control measures to balance its benefits with potential risks.

Total Dissolved Solids(TDS) refer to all organic and inorganic substances dissolved in a liquid such as minerals, salts, sugars and soluble proteins (Millar et al., 2016). From previous studies, TDS level can affect microbial metabolism, growth, and the spore formation features, then influencing the food products' longevity and quality (Kumar et al., 2021, Choi et al., 2014 and Hobson et al., 1999).

Milk protein powder is one of the main sources of TDS in dairy industries. Two primary types of milk powders are Skim Milk Powder (SMP) and Milk Protein Concentrate (MPC). The different protein and lactose content of MPC and SMP would result in different TDS contributions. During dairy processes such as evaporation and ultrafiltration, which would also greatly influence the total solids content. Research on how different TDS levels would affect the microorganisms is essential to optimise their beneficial uses and reduce the potential risks associated with it.

Biofilm is a community of microorganisms attached to the surface and embedded in a matrix of hydrated extracellular polymeric substances (Maria Pia Herrling et al., 2019). *Bacillus licheniformis* is generally considered safe and non pathogenic to humans. However, the biofilm of *Bacillus licheniformis* has been found able to grow in many temperature ranges

during milk powder processing industry and can cause food spoilage (Wang et al., 2021). Understanding how temperature could affect biofilms would also be important as it can inform sanitary control measures and help reduce contamination risks in food processing environments. Temperature variations can significantly influence biofilm development, structure which are key factors in maintaining food quality in the industry.

The aim of the thesis is to understand the biofilm formation, sporulation and spoilage enzyme production for *B.licheniformis* isolates from dairy manufacturing in different concentrations of total dissolved solids.

2.0 Literature Review

2.1 Introduction

Bacillus licheniformis is a spore-forming microorganism known for its special genetic background and safety and security features (He et al., 2023). It is a gram positive bacteria with spore forming ability (Rey et al., 2004). *B.licheniformis* has a great heat stability, high antimicrobial activity and stress resistance which makes it has a great potential in food manufacturing processes such as production of biological products, food waste treatment and probiotic functions. (He et al., 2023). It has adapted to produce metabolites and enzymes in food processing.

TDS, short for Total Dissolved Solids, refers to a measurement of the content of all inorganic and natural compounds in a fluid (Millar et al., 2016). It is vital for understanding the food products' microbial environment. As revealed by some research, the TDS level can affect microbial metabolism, growth, and the spore formation characteristics, then influencing the food products' longevity and quality (Kumar et al., 2021, Choi et al., 2014 and Hobson et al., 1999).

Milk powder is commonly used in the food industry and introduces Total Dissolved Solids (TDS) into products. Two primary types of milk powders are Skim Milk Powder (SMP) and Milk Protein Concentrate (MPC). SMP is produced by preheating skim milk, concentrating to 45% - 50% total solids by evaporation, heating concentrate and spray dried to SMP (Oldfield et al., 2005). SMP is characterised by having a low fat content (about 0.8%) and contains approximately 35% protein with 50% carbohydrate (Hoppe et al., 2008).

On the other side, MPC is produced by ultrafiltration of skim milk to concentrate the protein components before drying (Meena et al., 2020). MPC can contain protein levels in a range of 42% to 85% depending on the degree of concentration with correspondingly lower levels of lactose and minerals (Goulding et al., 2020). Understanding the TDS impact on the bacteria

helps to optimise their use in food processing while also contributing to managing spore forming bacteria in food products and processes.

The objectives of the review are to obtain a comprehensive understanding of the influence of TDS on metabolic rate of microbes, especially how various TDS degrees influence the sporulation process. Review the implications in the food sector and identify research gaps and future directions. Highlighting the lacking parts of the existing literary works and recommending for future research study that can provide deeper understanding into handling spore-forming bacteria in food and optimising the applications of *B.licheniformis* in food industries.

The aim of this review is to contribute to a comprehensive understanding of the biofilm of *B.licheniformis,* how enzymes are utilised and how TDS levels, as influenced by milk powder, can be managed to maximise the applications of *B.licheniformis* in food production and biotechnology.

2.2 Taxonomy of *B.licheniformis*

B.licheniformis is from the *Bacillus* genus and it belongs to the family *Bacillaceae* (He et al., 2023). They are characterised by the ability to produce endospores which are highly resistant to environmental extremities including heat, cold, dehydration and many disinfectants. This characteristic shows the genus' strong survival ability in extreme environments (He et al., 2023). The taxonomy of *B.licheniformis* is within the *B. subtilis* group and traditionally recognised in five physiologically similar species: *B.amyloliquefaciens*, *B.atrophaeus*, *B.licheniformis*, *B.pumilus*, and *B. subtilis*. These species have phenotypic similarities and can be distinguished through DNA homology studies and small subunit rRNA sequence comparisons, which shows their different but closely related nature (de Boer et al., 1994).

B.licheniformis shares an average of only 10% DNA homology with other members of the *B. subtilis* group, this makes it significantly different and especially for its closest relation to *B.amyloliquefaciens* which is 9-15% homology. This difference is also supported by biochemical tests as *B.licheniformis* strains uniquely show positive reactions for anaerobic growth, arginine dihydrolase production, starch hydrolysis and utilisation of propionate as a carbon source. These results set it apart from its group counterparts (de Boer et al., 1994).

Environmentally, *B.licheniformis* strains are widely present in most soils and notably dominating in nutrient-poor soils such as moorlands and deserts. They are also common in foods, especially the natural agricultural products where they likely colonise from wind-blown dust and soil particles. Their spores are also commonly found in processed or dried foods and herbs (de Boer et al., 1994) which indicates their widespread distribution.

As claimed by He et al., (2023), because of strong spore forming capacity and resilience in severe contexts, *B.licheniformis* acts as a crucial platform strain in the food synthetic biology. Food synthetic biology is an interdisciplinary area that combines biology and engineering to design and create new biological parts, devices and systems for applications in the food industry (Tyagi et al., 2014). This includes development of genetically engineered microorganisms to improve food production processes and create sustainable alternatives to traditional food sources (Lv et al., 2021). Given the biochemical and genetic versatility of *B.licheniformis*, it is the key in producing biologically active compounds like enzymes. Such a capacity is vital for large-scale application as well. According to He et al., (2023), this genus has heightened relevance in the environmental biology and biotechnological applications.

2.2 Role in Industry

Muras et al., (2021) state that due to innovations in genetic engineering, *B.licheniformis* has been applied in industrial procedures for producing diverse microbial compounds such as enzymes. Besides, technologies editing its DNA have significantly improved the efficiency of *B.licheniformis*. At the same time, particular strains such as WX-02 has been crucial in generating lots of indigenous and international healthy proteins like mannanase and nattokinase (Muras et al., 2021). Such a success is also precious in the food industry, in which the genetically tailored *B.licheniformis* pressures are designed to safely produce critical food enzymes like α-amylases (Muras et al., 2021).

Moreover, the application of *B.licheniformis* serves as a resource of various chemical activities. With unique thermostability and pH resistance, it is more useful than enzymes from other bacteria. This involves β-glucosidase, chitinase, endoglucanase, and xylase with strains separated from diverse contexts, showing the flexibility and effectiveness of the species (Muras et al., 2021). Due to the saprophytic way of life, *B.licheniformis* is more suitable for creating an optimal range of enzymes to degrade lipids, polysaccharides, proteins and different other substances. This emphasises its ability for various applications in the industry (Muras et al., 2021).

According to Muras et al., (2021), *B.licheniformis* is capable of generating antimicrobial, antifungal compounds, and the bioactive molecules with potential advantages. This involves the generation of biogenic selenium nanoparticles and exopolysaccharides with antiviral and immunoregulatory residential or commercial features. Beyond that, other usage contains incorporation of *B.licheniformis* in concrete to improve its stamina and application of its stress for biofuel manufacturing. All these underscore the potential of bacterium to make contributions to distinctive ranges in the sector (Muras et al., 2021).

2.3 Role in Food Market

2.3.1 Useful Enzymes in the Food Market

In the food industry, *B.licheniformis* has given a variety of practical enzymes that improve food flavour, appearance, and extend service life. Its enzymatic collection is well-suited for high-temperature processes which is additionally a typical requirement in food processing. For instance, the thermostable α-amylases and β-galactosidases from *B.licheniformis* can keep the high task under high temperatures, this applications in bread production to lower the viscosity of the dough and in dairy products manufacturing to transform lactose to more absorbable sugars (He et al., 2023). Urease and aglatoxin from *B.licheniformis* might likewise provide an improved preference in rice white wine and improved destruction of carcinogenic aflatoxins in foods, specifically.

Based on the research from (He et al., 2023). *B.licheniformis* is important in the food industry with its capacity for genetic and protein engineering. The development in the food sector shows progressively stringent needs on enzyme buildings such as catalytic performance and stability. This microbial is suitable to meet these demands and solidify its setting as the resource of food commercial enzymes.

2.3.2 Probiotic Applications

B.licheniformis also works as a probiotic, contributing to human health and wellness by boosting the digestive obstacle function and modulating the digestive tract microbiota. The application has been made use of and verified in different contexts consisting of the

avoidance of diarrhoea triggered by rotavirus infections and the mitigation of colitis impacts (He et al., 2023). *B.licheniformis* has also shown benefits in dental health and wellness and cognitive feature improvement, which shows its flexibility as a probiotic (He et al., 2023).

2.3.3 Food Biomanufacturing

From (He et al., 2023), In food manufacturing, *B.licheniformis* enhances the manufacturing of biomolecules such as antimicrobial peptides and flavour enhancers through metabolic engineering and fermentation optimization. By producing bacitracin, lichenysin and acetoin, it is conducive to improving food quality and security, implying its accessibility in the development of superior-quality food products.

2.4 *B.licheniformis* Spores

B.licheniformis has strong survival capacities through the production of endospores. These spores can resist detrimental environmental occasions. Because of the potential to survive in the food processes, they become a crucial issue in food quality (Berendsen et al., 2016). *B.licheniformis* spores are common in distinctive food ingredients and products, so food conservation techniques are needed to lower their risks in food production (Berendsen et al., 2016).

Given that *B.licheniformis* spores are highly resistant to chemical and physical treatments, it is essential to guarantee the inactivation or safeguarding against the spores' germination in food processing (Cho, 2020; Chung, 2020). As mentioned by Baril et al., (2012), the sporulation environment has greatly influenced the heat resistance of spores. This finding highlights the importance of understanding these environmental factors such as

temperature, nutrient availability and pH levels to prevent potential food quality issues in the industry.

Apart from that, Todorov et al. (2021) uncovered the *Bacillus* spores' resilience and biotechnological potential. By virtue of spore formation, *Bacillus* varieties can survive under extreme environments, which provides possibilities in the shipment and production of probiotic pressures. As revealed by clinical trials, *Bacillus* spores are capable of surviving and germinating in the human intestinal system, providing stimulating applications for human health and welfare (Todorov et al., 2021). Additionally, the use of spore-forming probiotics in animal manufacturing offers antibiotics a sustainable selection, highlighting the role of *Bacillus* spores in strengthening farming approaches (Todorov et al., 2021).

2.5 Spore Forming Microorganisms and Food Wastage

Spores creating microorganisms are regarded as the primary reason in the wastes of food like dairy and refrigerated meats (André et al., 2017). In previous research, the spores from *B.licheniformis* can survive at temperature levels of up to 100°C for several minutes (RODRIGUEZ et al., 1993; MONTVILLE & SAPERS, 1981). Due to such heat resistance capacity, this microorganism becomes a challenge in food protection and processing .

An example is the spore development capacity and the generation of enzymes from *B.licheniformis* have been regarded as a main spoilage factor in the bakery products (André et al., 2017). This can induce the changes of product in texture and odour. The spores from *B.licheniformis* are also related to the dairy products' spoilage, because their spores can survive through the food processing processes and then germinate under the best conditions and trigger the spoilage (André et al., 2017). As implied by the study, the spores from *B.licheniformis* and other thermophilic *Bacillus* varieties are generally found in milk powder.

The spoilage caused by *B.licheniformis* and other *Bacillus* categories presents the value of comprehending sporulation action and utilising particular tactics such as antimicrobial compounds to protect the food quality (André et al., 2017). Given the presence of these spore forming microbes in dairy processing, rigorous tracking and control procedures in production are needed to prevent perishing (André et al., 2017).

2.6 Aspects Impacting *Bacillus* Spore

2.6.1 Spore Formation

The sporulation process involves complex morphological changes that lead to the eventual release of fully developed spores. This is normally triggered by stress such as nutrient starvation or unfavourable environmental factors (Gauvry et al., 2017). Starting under nutrient constraint, the procedure brings about consecutive development phases and finally acquired resistance to extreme environmental issues (Cho, 2020; Chung, 2020). Such a developmental road is regulated by a cutting-edge network of transcription aspects induced by sensing unit kinases, underscoring the detailed rules of sporulation (Bressuire-Isoard et al., 2018).

Sporulation effectiveness and spore structure can be greatly affected by the ecological occasions, temperature, pH, and water activity. In fact, optimum growing conditions are favourable to optimum sporulation yield. However, these conditions' variances expand the sporulation procedure and may decrease yields (Bressuire-Isoard et al., 2018). Likewise, Melly et al., (2002) pointed out that the sporulation temperature exerts a significant impact on spore properties, involving resistance to diverse stress elements.

At the same time, biochemical variables are crucial. The core water content, levels of dipicolinic acid (DPA), and little, acid-soluble spore healthy proteins (SASP) are of great significance for spore's resistance and germination features. Particularly, spores prepared at distinctive temperature levels showcase similar numbers of DPA and SASP, despite changes of the core water web content and resistance to damp heat (Melly et al., 2002). Likewise, spores prepared in fluid or on agar plates present equivalent levels of DPA, core water, SASP, and individual coat proteins, but their resistance to heat and chemicals and their germination rates are different (Rose et al., 2007).

Factors like the sporulation supplementing nutrient level, particularly mono/di-valent cations, exerts great impact on sporulation return and spore safety. In addition, supplementing sporulation media with ions like Mn2+, Mg2+, Zn2+, and Ca2+ reinforces sporulation return and spore safety, and avoids spontaneous germination (Bressuire-Isoard et al., 2018). Apart from that, the nutritional structure of the sporulation tool affects the sporulation procedure and eventual spore yield, in which nutrients such as sugar elevate the spore yields (Bressuire-Isoard et al., 2018).

2.6.2 Spore Germination

Mortier et al. (2023) discovered the strength of *Bacillus subtilis* spores to protein aggregates (PA). Based on an inducible synthetic model, they demonstrated that the visibility of PAs in the sporulation procedure has not shown any negative effect on the spore's germination capacity and its subsequent survival under heat or UV stress. The research has shown that PAs can persist throughout the entire sporulation process after being encapsulated in forespore. The sporulation process also showed the ability to counter the disruptives caused

by protein aggregates and misfolded proteins and maintain functionality and resilience under stressful conditions. Understanding how *B. subtilis* and related species manage internal stressors would have significant implications for controlling spoilage causing organisms in the food industry.

Additionally, Trunet et al. (2020) assessed how the *Bacillus weihenstephanensis* and *B.licheniformis* spores' germination and outgrowth are influenced by the environment variables. While highlighting the sensitive extent of spore germination to suboptimal incubation issues, their research underscores a decline in the proportion of spores capable of germination as well as the germination time extension under non-ideal temperature. Through the usage of flow cytometry, it is possible to holistically examine the spores' physiological status under warm tension, and predict recuperation of spores after sublethal warmth treatments.

2.6.3 TDS Effects on Spore Production

Researching the impact of TDS on sporulation is important because it has significant implications for food quality and product shelf life. For example, Kumar et al. (2021) delved into the impact of TDS on spores and biofilm development in *Geobacillus stearothermophilus* stains A1, D1, P3, and ATCC 12980. Overall, the adaptive mechanism of *G.stearothermophilus* to different ecological situations is underscored in this research.

As pointed out by Kumar et al. (2021), an increase in TDS concentration remarkably postponed the onset of biofilm and spore development at a temperature of 55°C among all the assessed *G.stearothermophilus* stress. Moreover, this finding is especially applicable to the dairy industry, because *G.stearothermophilus* can form heat-resistant spores, form biofilms that are difficult to eliminate and may be of potential spoilage concern.

As suggested by the research results, the control of TDS concentration in dairy products processing contexts can help effectively diminish the threat of biofilm and spore formation by thermophilic bacteria. By increasing the TDS concentration, the beginning of observable biofilm and spore formation is delayed, utilising a potential home window during which preventive measures can be conducted more effectively.

Apart from that, the method of preconditioning stainless steel surface areas via adsorbed milk proteins can naturally raise the TDS of the surface and may even delay the onset of biofilm and spore formation. This approach suggests an unique and possibly considerable technique for boosting plant hygiene and minimising the threat of perishing brought on by *G. stearothermophilus* in milk powder manufacturing.

The ramifications of this research are substantial, offering a foundation for future research aimed at developing incorporated control techniques that take into consideration both the thermal processing specifications and the chemical composition of the handling atmosphere. Such methods could result in much more effective administration of thermophilic spore-formers in the dairy products market, therefore boosting item quality while reducing financial losses connected with putridity.

2.7 Biofilm

According to (Wang et al., 2021), although *Bacillus licheniformis* is not pathogenic, the biofilm is a significant challenge in the dairy industry due to their contribution to microbial contamination and food spoilage. Especially during evaporation processes of milk powder production where temperatures between 50℃ to 70℃ facilitate the bacterial multiplication. *B.licheniformis* is a facultative thermophile that forms biofilms in these high temperature regions in milk powder processing plants alongside obligate thermophiles such as *Geobacillus* and *Anoxybacillus*. *B.licheniformis* can produce biofilms at various interfaces including air-liquid interface, liquid solid interface and air-agar interface.

(Wang et al., 2021) Also found that amino acid biosynthesis, lipid, carbohydrate and nucleotide metabolism has been found to be very important during the first 8-12 hours of biofilm formation. This helps the biofilm to build and maintain the structure and be more resistant to environmental challenges such as cleaning and disinfection processes in factories.

Another research from (Wang et al., 2019) has pointed out that the metabolites of *Lactobacillus plantarum* can effectively delay the formation of *B.licheniformis* biofilm for up to 10 hours. The effect of cell free supernatants and pH control has reduced the adherence of *B.licheniformis* to stainless steel and glass surfaces. The metabolites have the ability to change the peptide content of the environment and inhibit the formation and stability of biofilm.

2.8 Conclusion

In conclusion, this review has indicated the importance of *Bacillus licheniformis* in the industry and how TDS level and other environmental factors that would affect the growth, sporulation and metabolism of other related bacteria. There are research gaps particularly in how different factors would affect those behaviour specifically for *B.licheniformis*. It is important to understand how these factors would affect the bacterium and the finding would contribute to enhancing food quality and extending product shelf life. This work may also help to optimise the use of the bacteria in other industry applications such as enzyme productions.

3.0 Objectives

- Determine the effect of Milk protein as Total Dissolved Solids on sporulation of *Bacillus licheniformis*

- Determine the biofilm formation of factory provided *Bacillus licheniformis* isolates in different temperature range

- Determine the Protease and Lipase activity of factory provided *B.licheniformis* and test heat sensitivity

4.0 Material and Methodology

Table 1. Isolate information

Isolate Number	Isolate Source
B1	Butter
B2	Butter
B3	Butter
MPC9	Milk Protein Concentrate
MPC10	Milk Protein Concentrate
MPC11	Milk Protein Concentrate
MPC12	Milk Protein Concentrate
MTSM1	Milk Treatment Skim Module
MTSM2	Milk Treatment Skim Module
MTSM3	Milk Treatment Skim Module
Isolate 55	Farm
Isolate 36	Farm
Isolate 42	Farm

4.1 Sporulation Measurement

4.1.1 Material

- *B.licheniformis* isolates MPC9, MPC12, B1, B2, MTSM1, MTSM2, MTSM3, 55, 36 and 42

- Gamma sterlised skim milk powder (SKM)
- Trypticase soy agar (TSA)
- Trypticase soy broth (TSB)
- Peptone water (PW)
- Water bath

4.1.2 Methodology

Direct plate count method has been used to determine the spore count of *B.licheniformis*. Fresh cultures of provided *Bacillus licheniformis* isolates were grown on TSA plates using streak plate method at 37℃ overnight. A single colony from each TSA plate was inoculated into TSB and incubated at 37℃ overnight to prepare liquid cultures.

Media with total dissolved solids (TDS) levels of 0%, 10%, 20% and 50% (w/v) were prepared by mixing gamma sterilised SKM with sterilised distilled water. Ten microliters (10uL) of the TSB culture were introduced into the prepared media and incubated at 37℃.

At intervals of 4, 8, 12, 16 hours, 10mL samples were taken from the cultures and heat treated in a water bath at 80℃ for 10 minutes to eliminate vegetative cells (Janštová & Lukášová, 2001). The heat treated samples were then diluted to 10^{-3} dilution with peptone water. Pour plate counts were performed using TSA to determine spore counts. Plates were incubated at 37℃ for 48 hours. The number of colonies counted after incubation was considered spore count.

Biofilm formation data were analysed using two way ANOVA. Minitab Statistical Software was used for the statistical analyses. A significance level of $p < 0.05$ was considered statistically significant.

4.2 Biofilm Screening

4.2.1 Material

- *B.licheniformis* isolates MPC9, MPC10, MPC11, MPC12, B1, B2, B3, MTSM1, MTSM2, MTSM3
- Falcon 96 well microtitre plate (Appendix 1)
- TSB
- TSA
- 98% Ethanol
- 0.5% crystal violet
- Vortex mixer
- BMG LABTECH SPECTROstar Nano Microplate Reader (Appendix 1)

4.2.2 Methodology

Crystal violet microtiter plate assay has been used for screening the biofilm. Procedures were based on information provided by the supervisor. (Appendix 5)

Fresh cultures of provided *Bacillus licheniformis* isolates were grown on TSA plates using streak plate method at 37℃ overnight. A single colony from each TSA plate was inoculated into TSB and incubated at 37℃ overnight to prepare liquid cultures. The cultures were diluted by transferring 100uL into 10mL of TSB media.

A volume of 200uL of diluted culture was inoculated into eight wells (one column) of a 96-well microtiter plate. The first column was filled with 200uL uninoculated TSB media as a negative control. Five microtiter plates were incubated at 20℃, 30℃, 37℃, 50℃ and 60℃ respectively for 18 hours statically to allow biofilm formation.

After incubation, the plates were washed three times with distilled water to remove media and dried at 40℃ for 30 minutes. Microtiter plate wells were stained with 200uL of 0.5% crystal violet solution for 15 minutes. Excess stain was removed by inverting the plate and followed by three washes with distilled water. Then the plates were dried at 40℃ for 30 minutes.

The crystal violet bound to biofilms was solubilised by adding 200uL of 98% ethanol to each well. The concentration of crystal violet which correlates with biofilm biomass was measured using BMG LABTECH SPECTROstar Nano Microplate Reader , with optical density has been set at 595 nm. The optical density readings difference between negative control and other isolates were used to determine the relative amount of biofilm formed by each isolate.

Biofilm formation data were analysed using one way ANOVA. Minitab Statistical Software was used for the statistical analyses. A significance level of $p < 0.05$ was considered statistically significant.

4.3 Enzymatic Activity

4.3.1 Material

- *B.licheniformis* isolates MPC9, MPC10, MPC11, MPC12, B1, B2, B3, MTSM1, MTSM2, MTSM3

- TSA

- TSB
- Milk Agar (Prepared by adding 60g SKM per L to plate count agar)
- Tributyrin agar
- Eppendorf Centrifuge 5702 (Appendix 2)
- 0.2um filter

4.3.2 Methodology

Fresh cultures of the provided *Bacillus licheniformis* isolates were grown on TSA plates using streak plate method at 37℃ overnight. Single colonies were picked and inoculated onto milk agar or tributyrin agar plates by drawing a line on the surface of agar. The plates were incubated at 37℃ and 30℃ for up to 72 hours and observed for positive results indicated by clear zones around culture. The clear zone indicates hydrolysis of milk protein by proteases on milk agar or hydrolysis of tributyrin by lipase on tributyrin agar. Non inoculated agar has been used as negative control. A triplicate test has been done for each isolate. Agar has been tested to work by testing known protease and lipase producing strains.

For isolates that showed positive enzymatic activity, fresh cultures grown on TSA plates (Streak Plate Method) were prepared for heat sensitivity tests. Single colonies were inoculated into 30mL TSB media and incubated for 48 to 72 hours. The cultures were then centrifuged at 4400 rpm for 20 minutes by using Eppendorf Centrifuge 5702. The supernatants were collected and filtered through 0.2 μm filters to remove residual cells.

The cell free supernatants were divided into four samples. Three samples were heat treated at 30 °C, 40 °C, and 50 °C, respectively. One untreated sample served as a control with unaffected enzymatic activity. Milk agar plates were prepared and a well on the agar was made using 1mL pipette tips. Each supernatant sample was inoculated into a well and incubated at 37℃ for 48 hours. The heat sensitivity of the protease was determined by

measuring and comparing the diameter of the clear zones formed around the wells which indicate hydrolysis of milk protein by protease enzymes.

5.0 Result and Discussion

5.1 Sporulation

The analysis includes spore count of 10 isolates across four TDS levels(0%, 10%, 20% and 50% w/v) in 16 hours. The isolates were sourced from butter (B1 and B2), milk protein concentrate (MPC 9 and MPC 12) and milk treatment skim module (MTSM 1- MTSM 3). A two way ANOVA analysis has shown a significant effect of both time and TDS level on sporulation across all samples, with p-values less than 0.05.

From graph observations in figure 1, isolates MTSM 1, MTSM 3, MPC 9 and MPC 12 have generally shown an increasing spore count with increased milk powder TDS level. Isolate MTSM1 has the most significant sporulation at higher TDS levels, especially at 50% as the standard error bar is clearly higher than all other TDS levels without any overlapping.

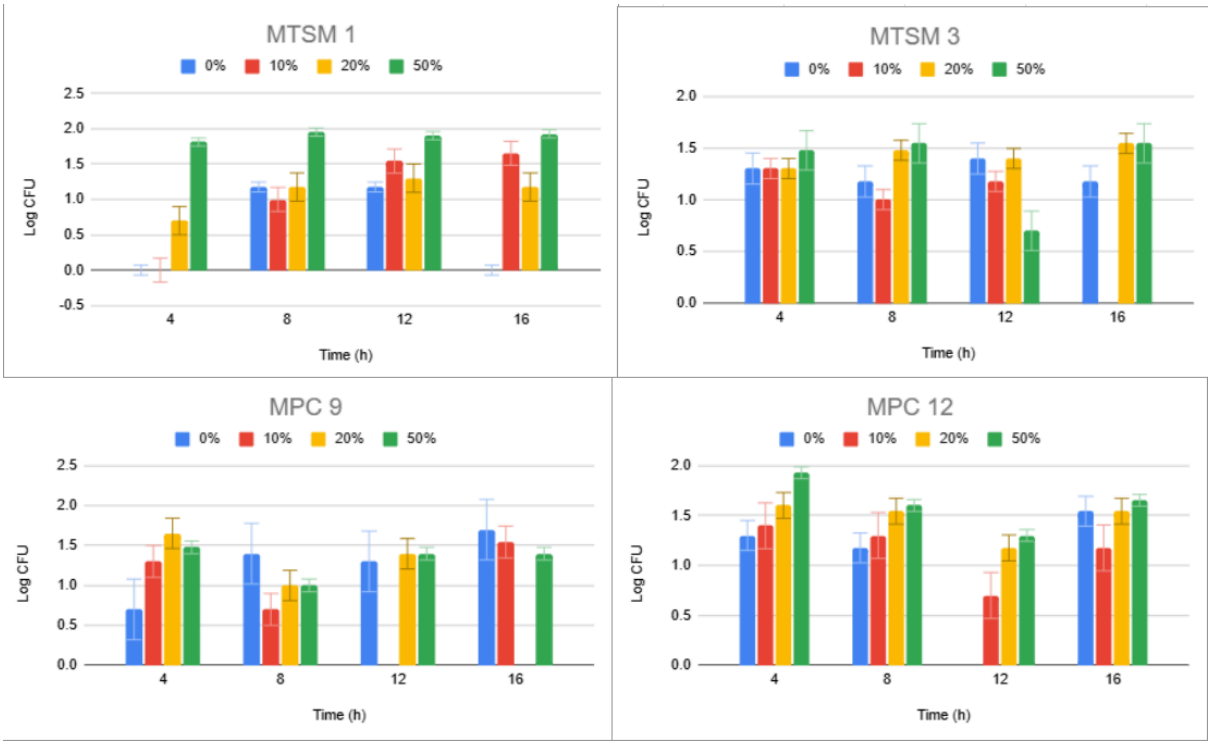

Figure 1. Spore Counts for Isolates MTSM 1, MTSM 3, MPC 9 and MPC 12

From figure 2, isolate MTSM 2 and B2 has shown no data reading during the test, this is due to formation of curds in the 50% milk media for isolates MTSM 2, this indicates the isolate were producing more acid during incubation in higher TDS level and causing curds in milk media. Other zero readings could be intolerance to the conditions or specific nutritional requirements not met by the milk powder concentrations used. Although there are frequent zero readings, isolate MTSM 2 were still clearly showing significant higher sporulation in increased TDS level (20%) with no overlapped standard error bar when compared with lower levels.

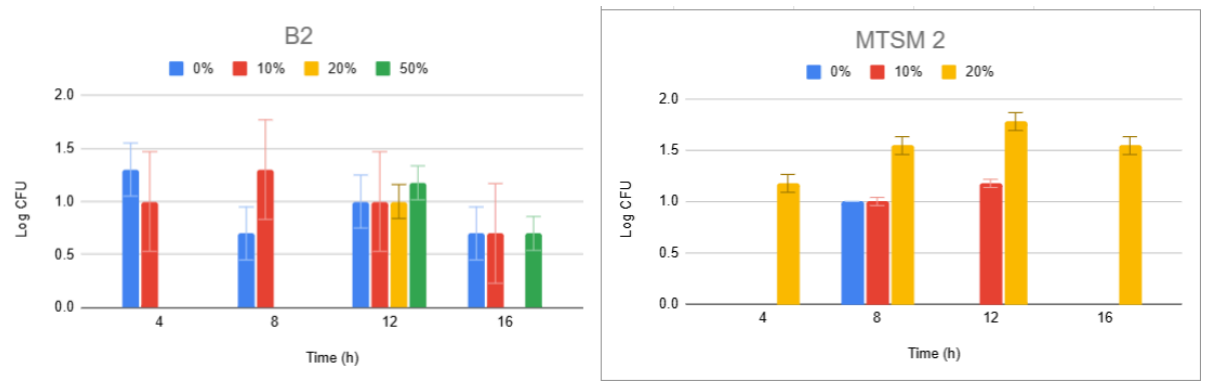

Figure 2. Spore Counts for Isolates B2 and MTSM 2

From figure 3, isolate B1 also shows curds in higher TDS level during measurement with highest sporulation showing on 10% TDS. This indicates the isolate was producing more acid at the highest TDS level (50%) and suggesting that moderate increase in TDS could promote sporulation on this isolate.

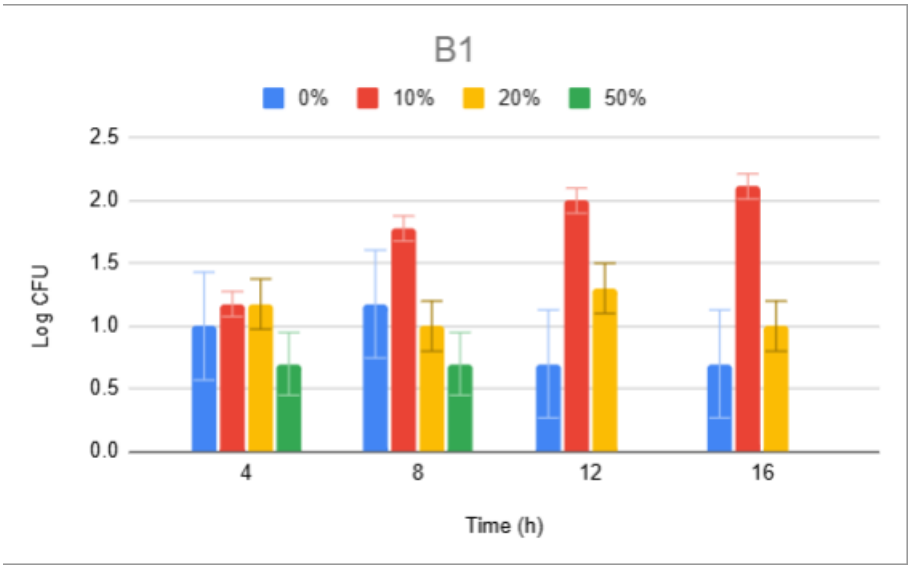

Figure 3. Spore Counts for Isolate B1

From farm sourced isolates (figure 4), isolate 55 and 36 has shown an early sporulation on lower TDS level 0% and 10%, with more overlapped error bars across all TDS levels. Isolate

42 has shown increased spore count in higher TDS levels which behaves similar to factory sourced isolates.

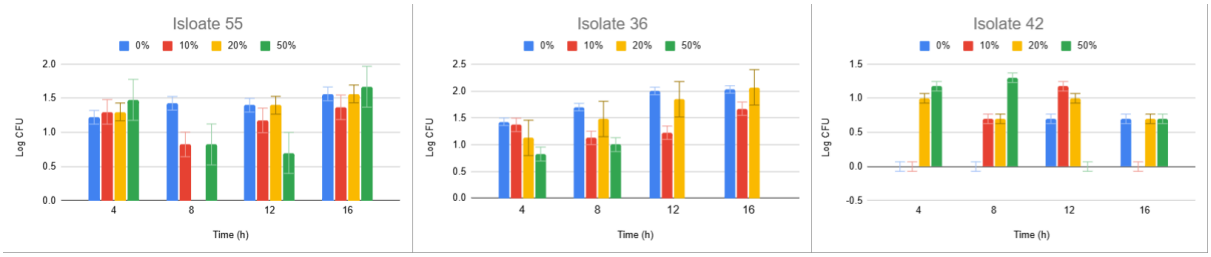

Figure 4. Spore Counts for Farm Sources Isolates

From the graphs, these isolates have shown different peak sporulation time and in different TDS levels, this indicates the isolates are having various metabolic and possibly genetic variations between.

Based on results, the farm sourced isolates have shown a varied adaptability in different TDS levels which is likely due to the fluctuating environmental conditions on the farm. The ability to handle different levels of nutrients in their environment makes it useful in unpredictable situations. The factory sourced isolates were performed more consistently in increased TDS levels. This is likely due to the fact that they are adapted to stable and nutrient-rich environments. This consistency in high TDS level makes the factory isolates can be harnessed in controlled industrial processes where nutrient levels are predictable and high.

Sporulation is usually associated with stress such as lower nutrient availability which is opposite to the result. (Fan et al., 2024) has shown that environmental factors like calcium ions can significantly affect biofilm formation by changing bacterial mobility, biofilm architecture and extracellular polymeric substance production. This indicates that ion specific effects can lead to complex bacterial responses. (Wang et al., 2021) noted that biofilm development depends on metabolic pathways including amino acid, lipid and carbohydrate metabolism. When these pathways have been disrupted such as high levels of certain ions, the biofilm is affected. This disruption may lead to stress-like conditions and

cause bacteria sporulation as a survival response. Therefore, high TDS levels may alter bacterial metabolism and prompting sporulation with environments that seem nutrient rich.

Frequent low spore count at 12 and 16 hour incubation may indicate the test procedure has potential deficiency. Other spore count methods such as flow cytometry are recommended for future study (Mathys et al., 2007). Flow cytometry is a technology that analyses characteristics of cells or particles as they flow past single or multiple lasers while suspended in solution. As each cell passes through, the detector measures light scattering and fluorescence characteristic of the stained cell and finally generates analysed data (McKinnon, 2018).

A vegetative cell and spore at 55℃(typical processing temperature) count is also recommended to improve the understanding how environmental factors (TDS) would affect the proportion of spore and vegetative cell. (Awasti et al., 2019) has studied *B.licheniformis* at lower temperatures (4.0 to 12.0℃), they found that the temperature would greatly affect the balance between vegetative cells and spores. The finding can help in controlling factors such as TDS and temperature to minimise spore formation and enhance the performance of processes like pasteurisation, since the bacteria in its vegetative form is more susceptible to pasteurisation.

5.2 Biofilm Formation

The analysis of the results for the measurement were focused on the effect of temperature (20,30,37,50 and 60℃) on biofilm growth of *Bacillus licheniformis* across 10 isolates from the milk factory. The isolates were sourced from butter (B1-B3), milk protein concentrate (P9-P12) and milk treatment skim module (T1-T3). One way ANOVA has been used to analyse the data.

Based on figure 5 to 7 and data analysis, all isolates have shown the biofilm formation has been significantly affected by temperatures with p-value < 0.05. By looking at the residual

plots, all samples have shown a generally good fit to the normal probability plot and histogram which indicates good reliability of confidence intervals and hypothesis tests. All samples have a moderate to low R^2 value which indicates that there are other factors not included in the model that may also be influencing the response variable. In result, isolates have shown different optimal growing temperatures while most isolates have highest growth in 30 to 50 ℃. All isolates have nearly zero growth at 20 and 60℃. This result has also been agreed with study from (Berendsen et al., 2016).

Isolate B1 and B2 has shown highest growth at 30℃, isolate T1, T2, B3 and P12 has highest growth on 37℃ and isolate T3, P9, P10 and P11 has the highest biofilm growth on 50℃. Isolate B2 and B3 have shown best temperature adaptation across 30 to 50℃ with generally high levels of growth without significant difference (highly overlapped confidence interval). From the graph, isolates P9 to P11 have all shown highest growth on 50℃ and P12 has a slightly lower growth. This indicates the isolates from MPC are all showing better adaptation on higher temperature ranges including 37℃ and 50℃.

However, isolate 9 from MPC has shown the lowest growth rate at 37℃ with higher growth at 30 and 50℃ which is an unexpected result. This could indicate some experimental variation or error present and replication of experiment is recommended to verify the reliability of the result. Another potential reason could be the methodology. According to (Kragh et al., 2019), the crystal violet assay tends to easily show substantial deviation from experiments to experiments even from well to well. They also found that the biomass development can differ stochastically, depending on the microenvironment within individual wells. To address the inconsistencies, a viable cell enumeration method (CFU/ml assay) used by (Fan et al., 2024) is recommended as an alternative. This method includes growing biofilm, detaching biofilm by using a mixer and measuring dislodged cell suspension by plate count. This method minimises the variability caused by microenvironmental factors within well and offers a more reliable and reproducible result. Using CFU counts can also help comparing results obtained from other assays and enhancing overall accuracy.

A previous study by (Yuan et al., 2022) has reported an OD_{594} value from 0.067 to 3.866. When compared with this measurement with OD_{595} value of 0.073 to 0.363, which are

significantly lower with much narrower range. The lower values in the measurement suggest that the biofilm formed under experimental conditions were less dense than those observed by Yuan et al. Although OD_{595} and OD_{594} are measured at slightly different wavelengths and may lead to slight absorbance differences. It is unlikely to cause the large disparity between two studies. As the result range still overlapped, it is more likely that the microbial samples in this study have inherently weaker biofilm forming capabilities.

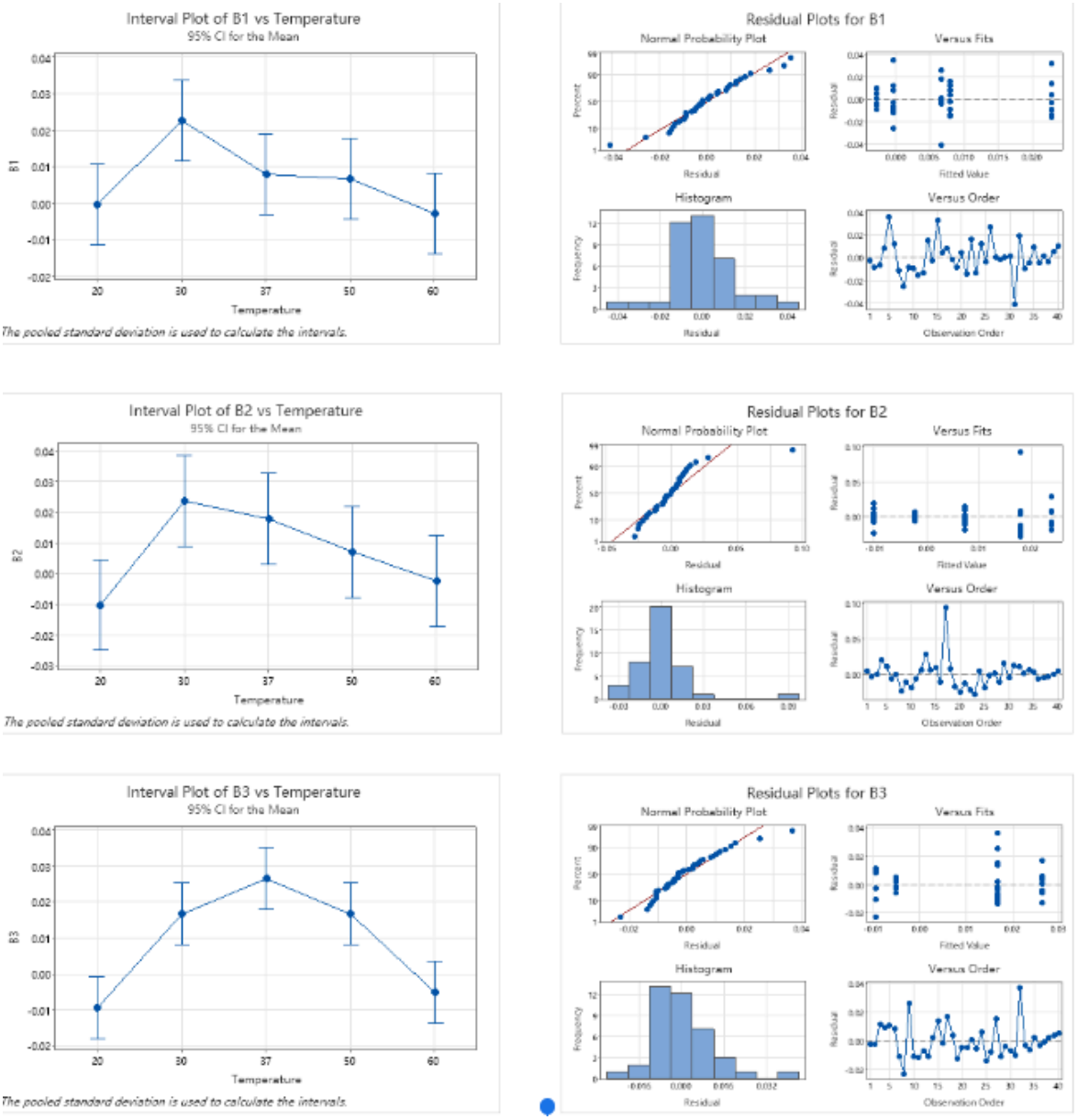

Figure 5. Interval Plots and Residual Plots for Isolates from Butter

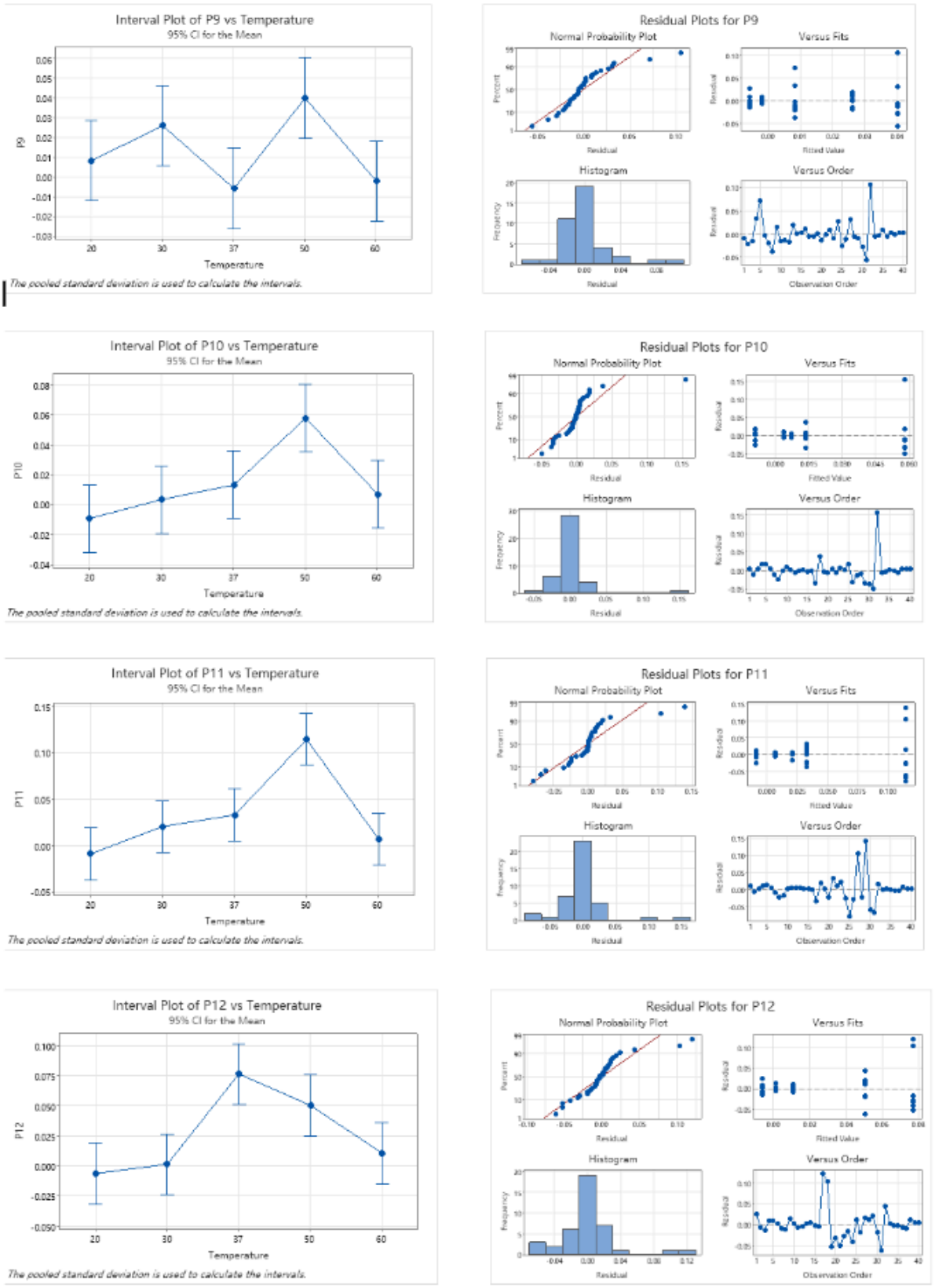

Figure 6. Interval Plots and Residual Plots for Isolates from MPC

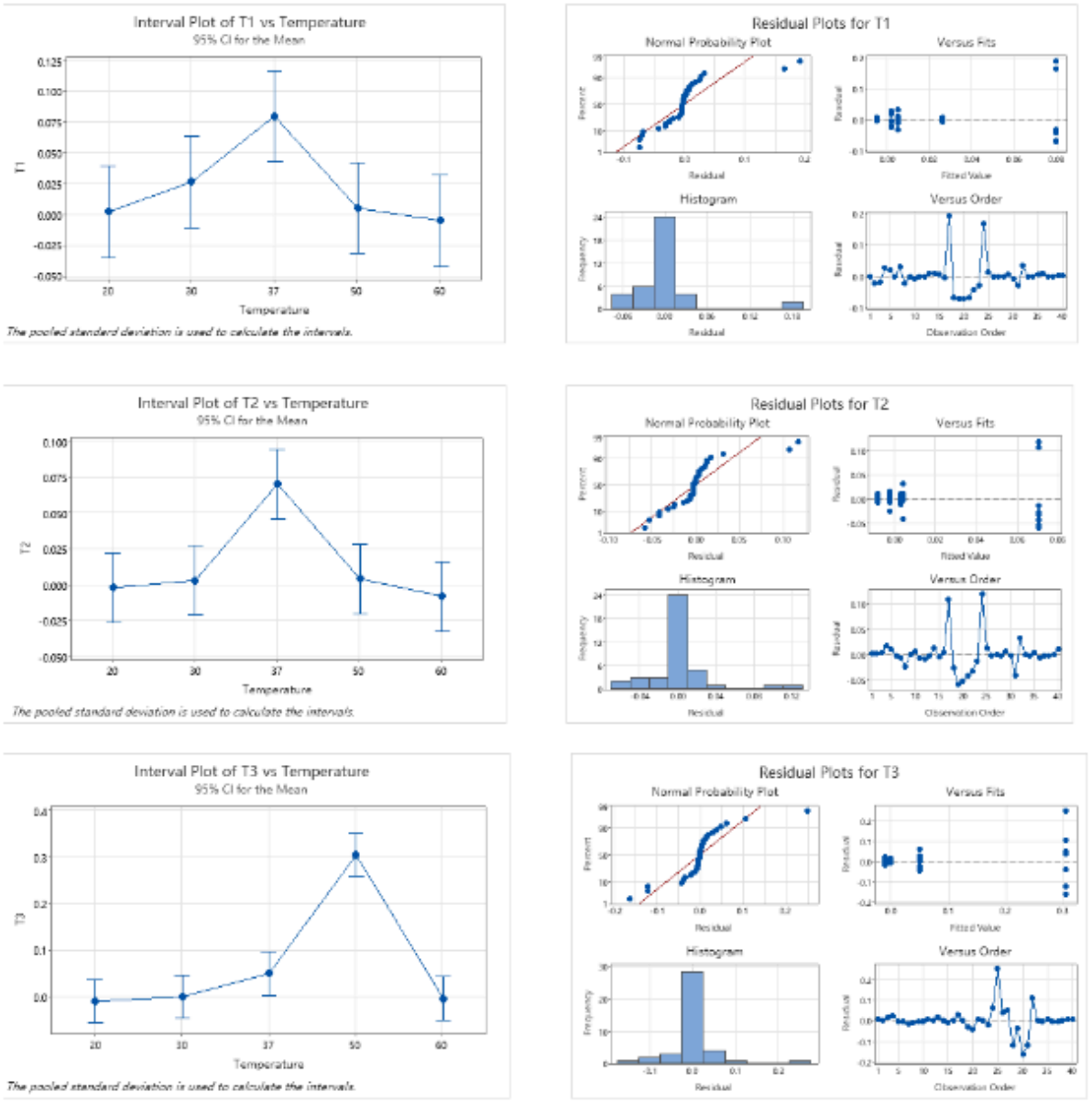

Figure 7. Interval Plots and Residual Plots for Isolates from Milk Treatment Skim Module

By relating the result to their potential living environment, isolates B1 and B2 showing highest growth at 30℃ could indicate an adaptation to lower temperature settings that are often used during butter solidification or storages. The wider temperature range adaptation

for B3 might suggest a genetic versatility making its survival in different temperature ranges during butter processing.

For MPC isolates (P9, P10, P11) showing highest growth at 50℃ and adaptation across higher temperature ranges (P12 slightly lower at 50℃), this might reflect a thermal adaptation at a higher temperature environment such as spray drying during production (Patel et al., 2020).

For isolates from milk treat skim modules, T1 and T2 has a typical growing curve with a peak at 37℃ and has not shown a clear growing response related to their potential environment. Isolate T3 has a growth peak at 50℃ might indicate heat adaptation during processes such as pasteurisation or some genetic versatility.

5.3 Enzymatic Activity

5.3.1 Screening

The enzymatic activity screening of *Bacillus licheniformis* was tested by inoculating colonies to milk and tributyrin agar plates and observing the formation of clear zones which indicate hydrolysis. Triplicate test has been performed to ensure the consistency and reliability of the result. In result (Table 1), only two isolates have shown positive results on protease test(clear zone refer to figure 8 to 10), no isolates have positive readings on lipase test. Both isolates have shown positive results at 37℃with over 48 hours incubating and for isolate T1 takes over 72 hours to observe positive results at 30℃. This observation indicates that there are potential differences in temperature sensitivity and enzymatic efficiency in the isolates.

Table 2. Enzyme Activity Test Result (Positive+, Negative-)

Protease			Lipase		
	30℃	37℃		30℃	37℃
B 1	-	-	B 1	-	-
B 2	-	-	B 2	-	-
B 3	-	+	B 3	-	-
MPC 9	-	-	MPC 9	-	-
MPC 10	-	-	MPC 10	-	-
MPC 11	-	-	MPC 11	-	-
MPC 12	-	-	MPC 12	-	-
MTSM 1	+	+	MTSM 1	-	-
MTSM 2	-	-	MTSM 2	-	-
MTSM 3	-	-	MTSM 3	-	-

Figure 8. Protease Hydrolysis Test of Isolate MTSM1 at 37℃ after 48 hour

Figure 9. Protease Hydrolysis Test of Isolate Butter 3 at 37℃ after 48 hour

Figure 10. Protease Hydrolysis Test of Isolate MTSM1 at 30℃ after 48 hour

5.3.2 Heat Sensitivity Test

Following the enzymatic activity screening, positive isolates were tested the stability and functionality of enzymes in temperature range include 30℃, 40℃ and 50℃ with untreated samples serving as control.

In the results of the heat sensitivity test, none of the isolates has produced a detectable clear zone across all temperature zones, including the control. This suggests the lack of enzymatic activity in the supernatant. This indicates that the proteases were either not secreted into the media or that the concentration was too low to hydrolyse the substrate in the agar.

According to (Bisswanger, 2014), it is essential to have proper concentration like substrates and enzymes to perform enzyme assays. To improve the result, techniques such as freeze thaw cycles and azocasein assay can be used.

Freeze thawing cycle is a physical technique which forms ice crystals that can puncture the cell membranes and helps to release cellular contents including enzymes. However, some enzymes might be sensitive to the temperature change during the cycle and lead to drops in activities, for some enzymes drops in the order of 30% can be observed.(Gagné, 2014)

Azocasein assay is a spectrometry method which works by measuring the azo (colouring component) released by hydrolysed casein (protein). Refer to (Teh et al., 2012), this assay includes preparing azocasein substrate, incubating and introducing enzyme sample to azocasein solution, stopping reaction using trichloroacetic acid (TCA) which precipitates unreacted proteins and finally measuring absorbance of supernatant at specific wavelength. The measurement result would be more sensitive to minimal concentration differences which can also potentially improve the result.

6. Conclusion

In sporulation analysis, a significant effect of both time and TDS levels on sporulation was observed across all samples (p-values <0.05). Factory sourced isolated has demonstrated increased spore counts with higher TDS levels which may indicate an adaptation to nutrient rich environments typical of industrial settings. Farm sourced isolates have shown varied adaptability to different TDS levels which likely reflect their exposure to fluctuating environmental conditions. The unexpected increase in sporulation at higher TDS levels suggests that increased ion concentrations may disrupt metabolic pathways and prompting sporulation even in nutrient rich conditions. Methods such as flow cytometry and measurements at processing temperatures (e.g., 55℃） are recommended to improve accuracy of future studies.

Biofilm formation analysis has shown temperature has significantly affected biofilm growth for all isolates with p-value < 0.05. Most isolates have shown an optimal biofilm formation from 30℃ to 50℃. Isolates from butter showed peak growth at 30℃ and 37℃ suggesting adaptation to lower temperatures which related to lower temperature during butter processing and storage. MPC isolates peaked at 50℃ reflect potential thermal adaptation to high temperature processes such as spray drying and concentrating. Some inconsistencies in the data may be due to experimental variability in crystal violet assay. Introducing more reliable methods like CFU counts could improve result consistency.

Enzymatic activity screening identified only two isolates with positive protease activity and none with lipase activity. Positive result was more readily after 48 hours incubation at 37℃ indicates temperature sensitivity in enzymatic efficiency. However, a heat sensitivity test showed no detectable result, suggesting that proteases were either not secreted into media or concentration was too low to hydrolyze the substrate. Techniques such as azocasein assay are recommended for more sensitive quantification.

Future research is recommended to focus on refining experimental methodologies to reduce variability and improve insights into metabolic and genetic factors influencing the bacterial behaviours.

Reference

Alegbeleye, O., Odeyemi, O. A., Strateva, M., & Stratev, D. (2022). Microbial spoilage of vegetables, fruits and cereals. Applied Food Research, 2(1), 100122. https://doi.org/10.1016/j.afres.2022.100122

André, S., Vallaeys, T., & Planchon, S. (2017). Spore-forming bacteria responsible for food spoilage. Research in Microbiology, 168(4), 379–387. https://doi.org/10.1016/j.resmic.2016.10.003

Awasti, N., Anand, S., & Djira, G. (2019). Sporulating behavior of *Bacillus licheniformis* strains influences their population dynamics during raw milk holding. Journal of Dairy Science, 102(7), 6001–6012. https://doi.org/10.3168/jds.2018-15613

Baril, E., Coroller, L., Couvert, O., Leguérinel, I., Postollec, F., Boulais, C., Carlin, F., & Mafart, P. (2012). Modeling heat resistance of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores as function of sporulation temperature and pH. Food Microbiology, 30(1), 29–36. https://doi.org/10.1016/j.fm.2011.09.017

Berendsen, E. M., Koning, R. A., Boekhorst, J., de Jong, A., Kuipers, O. P., & Wells-Bennik, M. H. J. (2016). High-Level Heat Resistance of Spores of *Bacillus amyloliquefaciens* and *Bacillus licheniformis* Results from the Presence of a spoVA Operon in a Tn1546 Transposon. Frontiers in Microbiology, 7. https://doi.org/10.3389/fmicb.2016.01912

Bisswanger, H. (2014). Enzyme Assays. Perspectives in Science, 1(1-6), 41–55. https://doi.org/10.1016/j.pisc.2014.02.005

Bressuire-Isoard, C., Broussolle, V., & Carlin, F. (2018). Sporulation environment influences spore properties in *Bacillus*: evidence and insights on underlying molecular and physiological mechanisms. FEMS Microbiology Reviews, 42(5), 614–626. https://doi.org/10.1093/femsre/fuy021

Cho, W.-I., & Chung, M.-S. (2020). *Bacillus* spores: a review of their properties and inactivation processing technologies. Food Science and Biotechnology, 29(11), 1447–1461. https://doi.org/10.1007/s10068-020-00809-4

Choi, J. S., Kim, J. T., & Joo, H. J. (2014). Effect of Total Dissolved Solids Injection on Microbial Diversity and Activity Determined by 16S rRNA Gene Based Pyrosequencing and Oxygen Uptake Rate Analysis. Environmental Engineering Science, 31(8), 474–480. https://doi.org/10.1089/ees.2014.0043

de Boer, A. S., Priest, F., & Diderichsen, B. (1994). On the industrial use of *Bacillus licheniformis*: a review. Applied Microbiology and Biotechnology, 40(5), 595–598. https://doi.org/10.1007/bf00173313

Fan, L., Dai, H., Zhou, W., Yuan, L., Yang, J., Yang, Z., & Jiao, X. (2024). Unraveling the significance of calcium as a biofilm promotion signal for *Bacillus licheniformis* strains isolated from dairy products. Food Research International, 182, 114145. https://doi.org/10.1016/j.foodres.2024.114145

Fan, L., Liu, S., Dai, H., Yuan, L., Yang, Z., & Jiao, X. (2024). Genotype-phenotype evaluation of the heterogeneity in biofilm formation by diverse *Bacillus licheniformis* strains isolated from dairy products. International Journal of Food Microbiology, 416, 110660. https://doi.org/10.1016/j.ijfoodmicro.2024.110660

Gagné, F. (2014). Tissue Preparation and Subcellular Fractionation Techniques. Biochemical Ecotoxicology, 21–31. https://doi.org/10.1016/b978-0-12-411604-7.00002-7

Gauvry, E., Mathot, A.-G., Leguérinel, I., Couvert, O., Postollec, F., Broussolle, V., & Coroller, L. (2017). Knowledge of the physiology of spore-forming bacteria can explain the origin of spores in the food environment. Research in Microbiology, 168(4), 369-378. https://doi.org/10.1016/j.resmic.2016.10.006

Goulding, D. A., Fox, P. F., & O'Mahony, J. A. (2020). Milk proteins: An overview. Milk Proteins, 21–98. https://doi.org/10.1016/b978-0-12-815251-5.00002-5

He, H., Yu, Q., Ding, Z., Zhang, L., Shi, G., & Li, Y. (2023). Biotechnological and food synthetic biology potential of platform strain: *Bacillus licheniformis*. Synthetic and Systems Biotechnology, 8(2), 281–291. https://doi.org/10.1016/j.synbio.2023.03.008

Hobson, P., Burch, M., & Fallowfield, H. J. (1999). Effect of total dissolved solids and irradiance on growth and toxin production by Nodularia spumigena , 11(6), 551–558. https://doi.org/10.1023/a:1008193918189

Hoppe, C., Andersen, G. S., Jacobsen, S., Mølgaard, C., Friis, H., Sangild, P. T., & Michaelsen, K. F. (2008). The Use of Whey or Skimmed Milk Powder in Fortified Blended Foods for Vulnerable Groups. The Journal of Nutrition, 138(1), 145S161S. https://doi.org/10.1093/jn/138.1.145S

Janštová, B., & Lukášová, J. (2001). Heat Resistance of *Bacillus* spp. Spores Isolated from Cow's Milk and Farm Environment. Acta Veterinaria Brno, 70(2), 179–184. https://doi.org/10.2754/avb200170020179

Kumar, M., Flint, S., Palmer, J., Chanapha, S., & Hall, C. (2021). Influence of Incubation Temperature and Total Dissolved Solids on Biofilm and Spore Formation by Dairy Isolates of *Geobacillus stearothermophilus*. Applied and Environmental Microbiology, 87(8). https://doi.org/10.1128/aem.02311-20

Lv, X., Wu, Y., Gong, M., Deng, J., Gu, Y., Liu, Y., Li, J., Du, G., Ledesma-Amaro, R., Liu, L., & Chen, J. (2021). Synthetic biology for future food: Research progress and future directions. Future Foods, 3, 100025. https://doi.org/10.1016/j.fufo.2021.100025

Maria Pia Herrling, Lackner, S., Nirschl, H., Horn, H., & Guthausen, G. (2019). Recent NMR/MRI studies of biofilm structures and dynamics. Annual Reports on NMR Spectroscopy, 163–213. https://doi.org/10.1016/bs.arnmr.2019.02.001

Mathys, A., Chapman, B. E., Bull, M. J., Heinz, V., & Knorr, D. (2007). Flow cytometric assessment of *Bacillus* spore response to high pressure and heat. 8(4), 519–527. https://doi.org/10.1016/j.ifset.2007.06.010

McKinnon, K. M. (2018). Flow Cytometry: an Overview. Current Protocols in Immunology, 120(1), 5.1.1–5.1.11. https://doi.org/10.1002/cpim.40

Meena, G. S., Singh, A. K., & Gupta, V. K. (2020). Production and characterization of cow milk based low-protein milk protein concentrate (MPC) powders. Journal of Food Science and Technology. https://doi.org/10.1007/s13197-020-04824-5

Melly, E., Genest, P. C., Gilmore, M. E., Little, S., Popham, D. L., Driks, A., & Setlow, P. (2002). Analysis of the properties of spores of *Bacillus subtilis* prepared at different temperatures. Journal of Applied Microbiology, 92(6), 1105–1115. https://doi.org/10.1046/j.1365-2672.2002.01644.x

Millar, G. J., Couperthwaite, S. J., & Moodliar, C. D. (2016). Strategies for the management and treatment of coal seam gas associated water. Renewable and Sustainable Energy Reviews, 57, 669–691. https://doi.org/10.1016/j.rser.2015.12.087

MONTVILLE, T. J., & SAPERS, G. M. (1981). Thermal Resistance of Spores from pH, Elevating Strains of *Bacillus licheniformis*. Journal of Food Science, 46(6), 1710–1712. https://doi.org/10.1111/j.1365-2621.1981.tb04469.x

Mortier, J., Cambré, A., Schack, S., Christie, G., & Aertsen, A. (2023). Impact of Protein Aggregates on Sporulation and Germination of *Bacillus subtilis*. Microorganisms, 11(9), 2365–2365. https://doi.org/10.3390/microorganisms11092365

Muras, A., Romero, M., Mayer, C., & Otero, A. (2021). Biotechnological applications of *Bacillus licheniformis*. Critical Reviews in Biotechnology, 41(4), 609–627. https://doi.org/10.1080/07388551.2021.1873239

Oldfield, D. J., Taylor, M. W., & Singh, H. (2005). Effect of preheating and other process parameters on whey protein reactions during skim milk powder manufacture. International Dairy Journal, 15(5), 501–511. https://doi.org/10.1016/j.idairyj.2004.09.004

Patel, A. S., Yeung, C. K., Brennan, C., & Zheng, H. (2020). Ingredients & Formulation. Reference Module in Food Science. https://doi.org/10.1016/b978-0-12-818766-1.00161-6

Rey, M. W., Ramaiya, P., Nelson, B. A., Brody-Karpin, S. D., Zaretsky, E. J., Tang, M., de Leon, A., Xiang, H., Gusti, V., Clausen, I. G., Olsen, P. B., Rasmussen, M. D., Andersen, J. T., Jørgensen, P. L., Larsen, T. S., Sorokin, A., Bolotin, A., Lapidus, A., Galleron, N., & Ehrlich, S. D. (2004). Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. Genome Biology, 5(10), r77. https://doi.org/10.1186/gb-2004-5-10-r77

RODRIGUEZ, J. H., COUSIN, M. A., & NELSON, P. E. (1993). Thermal Resistance and Growth of *Bacillus licheniformis* and *Bacillus subtilis* in Tomato Juice. Journal of Food Protection, 56(2), 165–168. https://doi.org/10.4315/0362-028x-56.2.165

Rose, R., Setlow, B., Monroe, A., Mallozzi, M., Driks, A., & Setlow, P. (2007). Comparison of the properties of *Bacillus subtilis* spores made in liquid or on agar plates. Journal of Applied Microbiology, 103(3), 691–699. https://doi.org/10.1111/j.1365-2672.2007.03297.x

Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., & Lindsay, D. (2012). Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. International Journal of Food Microbiology, 157(1), 28–34. https://doi.org/10.1016/j.ijfoodmicro.2012.04.008

Todorov, S. D., Ivanova, I. V., Popov, I., Weeks, R., & Chikindas, M. L. (2021). *Bacillus* spore-forming probiotics: benefits with concerns?. Critical Reviews in Microbiology, 48(4), 513–530. https://doi.org/10.1080/1040841x.2021.1983517

Trunet, C., Mtimet, N., Mathot, A-G., Postollec, F., Leguerinel, I., Couvert, O., Broussolle, V., Carlin, F., & Coroller, L. (2020). Suboptimal *Bacillus licheniformis* and *Bacillus weihenstephanensis* spore incubation conditions increase heterogeneity of spore outgrowth time. Applied and Environmental Microbiology. https://doi.org/10.1128/aem.02061-19

Tyagi, A., Kumar, A., Aparna, S. V., Mallappa, R. H., Grover, S., & Batish, V. K. (2014). Synthetic Biology: Applications in the Food Sector. Critical Reviews in Food Science and Nutrition, 56(11), 1777–1789. https://doi.org/10.1080/10408398.2013.782534

Wang, N., Gao, J., Yuan, L., Jin, Y., & He, G. (2021). Metabolomics profiling during biofilm development of *Bacillus licheniformis* isolated from milk powder. International Journal of Food Microbiology, 337, 108939. https://doi.org/10.1016/j.ijfoodmicro.2020.108939

Yuan, L., Fan, L., Liu, S., Sant'Ana, A. S., Zhang, Y., Zhou, W., Zheng, X., He, G., Yang, Z., & Jiao, X. (2022). Bacterial community analysis of infant foods obtained from Chinese markets by combining culture-dependent and high-throughput sequence methods. Food Research International, 162, 112060–112060. https://doi.org/10.1016/j.foodres.2022.112060

Appendix

1. Biofilm Materials

Figure 11. Photo of Falcon 96 well microtitre plate

Figure 12. BMG LABTECH SPECTROstar Nano Microplate Reader

2. Enzymatic Heat Sensitivity Test Material

Figure 13. Eppendorf Centrifuge 5702

3. Spore Count Data Analysis

Raw data were recorded by calculating the average value of three plate count

Table 2. Raw Data and Calculated Log CFU for spore counts of 10 isolates

MTSM1	0%	10%	20%	50%
4	0	0	5	65
8	15	10	15	90
12	15	35	20	80
16	0	45	15	85
Log CFU	0%	10%	20%	50%
4	0	0	0.6989700043	1.812913357
8	1.176091259	1	1.176091259	1.954242509
12	1.176091259	1.544068044	1.301029996	1.903089987
16	0	1.653212514	1.176091259	1.929418926

MTSM2	0%	10%	20%	50%
4	0	0	15	Curd
8	10	10	35	Curd
12	0	15	60	Curd
16	0	0	35	Curd
Log CFU	0%	10%	20%	50%
4	#NUM!	#NUM!	1.176091259	#VALUE!
8	1	1	1.544068044	#VALUE!
12	#NUM!	1.176091259	1.77815125	#VALUE!
16	#NUM!	#NUM!	1.544068044	#VALUE!

Butter 1	0%	10%	20%	50%
4	10	15	15	5
8	15	60	10	5
12	5	100	20	0
16	5	130	10	0
Log CFU	0%	10%	20%	50%
4	1	1.176091259	1.176091259	0.6989700043
8	1.176091259	1.77815125	1	0.6989700043
12	0.6989700043	2	1.301029996	#NUM!
16	0.6989700043	2.113943352	1	#NUM!

Butter 2	0%	10%	20%	50%
4	20	10	0	0
8	5	20	0	0
12	10	10	10	15
16	5	5	0	5
Log CFU	0%	10%	20%	50%
4	1.301029996	1	#NUM!	#NUM!
8	0.6989700043	1.301029996	#NUM!	#NUM!
12	1	1	1	1.176091259
16	0.6989700043	0.6989700043	#NUM!	0.6989700043

MTSM3	0%	10%	20%	50%
4	2	2	2	3
8	15	10	30	35
12	25	15	25	5
16	15	Curd	35	35
Log CFU	0%	10%	20%	50%
4	0.3010299957	0.3010299957	0.3010299957	0.4771212547
8	1.176091259	1	1.477121255	1.544068044
12	1.397940009	1.176091259	1.397940009	0.6989700043
16	1.176091259	#VALUE!	1.544068044	1.544068044

MPC9	0%	10%	20%	50%
4	5	20	45	30
8	25	5	10	10
12	20	0	25	25
16	50	35	0	25
Log CFU	0%	10%	20%	50%
4	0.6989700043	1.301029996	1.653212514	1.477121255
8	1.397940009	0.6989700043	1	1
12	1.301029996	#NUM!	1.397940009	1.397940009
16	1.698970004	1.544068044	#NUM!	1.397940009

55	0%	10%	20%	50%
4	16.66666667	2	2	3
8	26.66666667	6.666666667	0	6.666666667
12	25	15	25	5
16	36.66666667	23.33333333	36.66666667	46.66666667
Log CFU	0%	10%	20%	50%
4	1.22184875	0.3010299957	0.3010299957	0.4771212547
8	1.425968732	0.8239087409	#NUM!	0.8239087409
12	1.397940009	1.176091259	1.397940009	0.6989700043
16	1.56427143	1.367976785	1.56427143	1.669006781

36	0%	10%	20%	50%
4	26.66666667	23.33333333	13.33333333	6.666666667
8	50	13.33333333	30	10
12	100	16.66666667	70	0
16	106.6666667	46.66666667	116.6666667	0
Log CFU	0%	10%	20%	50%
4	1.425968732	1.367976785	1.124938737	0.8239087409
8	1.698970004	1.124938737	1.477121255	1
12	2	1.22184875	1.84509804	#NUM!
16	2.028028724	1.669006781	2.06694679	#NUM!

MPC 12	0%	10%	20%	50%
4	20	25	40	85
8	15	20	35	40
12	0	5	15	20
16	35	15	35	45
Log CFU	0%	10%	20%	50%
4	1.301029996	1.397940009	1.602059991	1.929418926
8	1.176091259	1.301029996	1.544068044	1.602059991
12	#NUM!	0.6989700043	1.176091259	1.301029996
16	1.544068044	1.176091259	1.544068044	1.653212514

42	0%	10%	20%	50%
4	0	0	10	15
8	0	5	5	20
12	5	15	10	0
16	5	0	5	5
Log CFU	0%	10%	20%	50%
4	0	0	1	1.176091259
8	0	0.6989700043	0.6989700043	1.301029996
12	0.6989700043	1.176091259	1	0
16	0.6989700043	0	0.6989700043	0.6989700043

4. Biofilm Data Analysis

1. Calculate average value of absorbance of control column

2. Calculate average value of (culture column - average value of control column)

3. Calculate standard deviation of each temperature (Using Excel Formula STDEV.S)

4. Calculate standard error using formula SE = STDEV / Sqrt 8(number of sample)

Table 3. Calculated Data for Biofilm Analysis

Temperature	T2	STDEV	SE	Temperature	T3	STDEV	SE
20.000	0.000	0.015	0.005	20.000	0.000	0.020	0.007
30.000	0.003	0.014	0.005	30.000	0.000	0.013	0.005
37.000	0.069	0.075	0.027	37.000	0.032	0.038	0.014
50.000	0.013	0.030	0.011	50.000	0.136	0.153	0.054
60.000	0.005	0.009	0.003	60.000	0.003	0.007	0.003
Temperature	B3	STDEV	SE	Temperature	P9	STDEV	SE
20.000	0.000	0.017	0.006	20.000	0.008	0.040	0.014
30.000	0.017	0.020	0.007	30.000	0.026	0.020	0.007
37.000	0.010	0.017	0.006	37.000	0.010	0.016	0.006
50.000	0.018	0.035	0.012	50.000	0.041	0.058	0.020
60.000	0.003	0.007	0.003	60.000	0.004	0.008	0.003
Temperature	P12	STDEV	SE	Temperature	MTSM 1	STDEV	SE
20.000	0.000	0.020	0.007	20.000	0.002	0.010	0.008
30.000	0.002	0.013	0.005	30.000	0.026	0.003	0.007
37.000	0.072	0.078	0.028	37.000	0.108	0.041	0.006
50.000	0.023	0.039	0.014	50.000	0.015	0.011	0.009
60.000	0.006	0.011	0.004	60.000	0.005	0.003	0.003
Temperature	B1	STDEV	SE	Temperature	B2	STDEV	SE
20.000	0.000	0.024	0.008	20.000	0.000	0.016	0.006
30.000	0.023	0.021	0.007	30.000	0.024	0.017	0.006
37.000	0.012	0.018	0.006	37.000	0.042	0.049	0.017
50.000	0.008	0.025	0.009	50.000	0.021	0.038	0.013
60.000	0.005	0.009	0.003	60.000	0.005	0.009	0.003
Temperature	P10	STDEV	SE	Temperature	P11	STDEV	SE
20.000	0.000	0.017	0.006	20.000	0.000	0.014	0.005
30.000	0.004	0.013	0.005	30.000	0.021	0.012	0.004
37.000	0.018	0.024	0.009	37.000	0.025	0.032	0.011
50.000	0.062	0.079	0.028	50.000	0.074	0.090	0.032
60.000	0.003	0.007	0.002	60.000	0.004	0.008	0.003

5. Crystal Violet Assay

Microtitre plate method – biofilm screening

For each culture to be testing, 100 uL a fresh overnight culture prepared in TSB media at 37 °C is transferred to 10 mL of fresh sterile TSB medium and mixed on a vortex mixer.

For each diluted culture above, 8 wells of a 96-well polystyrene plates (Falcon® 96, 35 3072, Becton, Dickinson & Company, USA) are inoculated with 0.200 mL of culture. The plate should include included 8 negative control wells, which comprise 0.200 mL of uninoculated media.

Biofilm growth

In order to allow the growth of biofilms, the microtitre plates are covered with a lid and incubated at 37C for 18 h.

Biofilm readings

In order to measure the amount of biofilm forming on the plates after incubation, the plates are washed three times with sterile distilled water, and dried at 42°C for 30 minutes. After drying, the biofilm in the wells are stained with 0.200 mL of 0.5% of crystal violet and left on the bench for 15 minutes. The crystal violet is removed by inverting the plates over a sink, and the wells are washed three times with sterile distilled water and dried for another 30 minutes at 42°C. The stain that has been taken up by the biofilm cells on the surface of the plate is released from the biofilm in the wells by adding 0.200 ml of 98% ethanol. The concentration of the crystal violet in the ethanol is used to determine the relative amount of biofilm by measuring the optical density at 595 nm using a microtitre plate reader (ELx808 Ultra Microplate Reader, Bio-tek instruments, Inc.)