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Characterisation of proteases and lipases produced by *Paenibacillus* spp. and analysis of heat stability of these enzymes

‘A thesis presented in partial fulfilment of the requirements for the Master of Food technology at Massey University, Manawatū, New Zealand’hea

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Abstract

The aim of this study includes the isolation of *Paenibacillus* species from raw milk, investigation of *Paenibacillus* species that produces protease and lipase, investigation of the effect of temperature, growth medium and inoculum conditions (fresh and chilled) on the production of protease and lipase by *Paenibacillus* species and the heat stability of protease and lipase produced by *Paenibacillus* species. The isolation of *Paenibacillus* species from raw milk was carried out by using milk plate count agar and the isolated species were identified using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysisThere were none *Paenibacillus* species isolated from raw milk therefore pre-isolated *Paenibacillus* species obtained from Massey University microbiology lab culture collection were used for further experiments. The production of protease and lipase were tested using milk agar and tributyrin agar plates respectively. Seven out of 26 isolates were strong positive for protease at 37 °C. Only 2 isolates were positive for proteolysis at 30 °C and 2 isolates were strongly positive for proteolysis at 30 °C. The effects of inoculum conditions (fresh and chilled) were tested by comparing the ability of isolated *Paenibacillus* species at day 1 (fresh) and day 31 (chilled) of storage and there were no obvious differences. The effect of medium was tested by growing *Paenibacillus* in TSB, 10% TSB and 1% skim milk. *Paenibacillus* produced more proteolysis in full strength Tryptic Soy Broth (TSB), 10% TSB and 1% skim milk. The enzyme activities were further investigated by using azocasein and p-nitrophenol palmitate assays. Seven isolates produced obvious amounts of proteolysis, and the highest amount of proteolysis produced was 72 U/mL. There was no proteolysis and lipolysis found after heat treatment at 100 °C for 10 minutes which indicated no production of heat-stable enzymes by the *Paenibacillus* isolates. In conclusion, the investigation of enzyme activities of

species provides new insights for the dairy industry with no heat-stable enzymes produced by the *Paenibacillus* isolates tested. Further studies are required for medium effect on enzyme production and heat stability of enzyme produced by testing different medium of growth and different temperature conditions for the enzyme produced.

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1. Introduction

This study explores the enzymatic activities of *Paenibacillus* isolates for their ability to produce proteases and lipases where these enzymes are crucial in industrial and dairy contexts. *Paenibacillus* spp. are gram-positive, rod-shaped, and endospore-forming bacteria with diverse metabolic capabilities. These bacteria can be found in environments ranging from soil to dairy products and can influence food quality through spoilage or preservation. The study was carried out to isolate *Paenibacillus* spp. from raw milk, investigate the conditions affecting enzyme production, and assess the heat stability of these enzymes. While enzyme activities like proteolysis were observed in certain isolates under specific conditions, the previous studies found (Trmčić et al., 2015) no evidence of heat-stable enzymes produced by *Paenibacillus* spp. The findings by this study will provide insights into the potential characteristics and challenges of using enzymes produced by *Paenibacillus* in the dairy industry to provide the need for further exploration into growth medium and thermal processing conditions.

The aims of the study were to isolate *Paenibacillus* from raw milk, determining their capacity to produce protease and lipase through lipid and protein hydrolysis assays, and to evaluate the effects of temperature, medium and inoculum conditions (fresh and chilled) on protease and lipase production. The heat stability of these enzymes was examined to determine if they are likely to survive industrial processes, particularly in thermally intensive applications.

2. Literature review

2.1 Introduction

The *Paenibacillus* genus is a group of gram-positive bacteria, rod shape, facultative anaerobic and endospore forming. It was initially included within the *Bacillus* genus and was reclassified according to their distinct phylogenetic characteristics. *Paenibacillus* isolates can grow in diverse environments ranging from soil and water to extreme habitats. Their metabolic and physiological activities vary by adapting to different environments. *Paenibacillus* spp. are studied in agriculture where they enhance soil fertility and plant health through nitrogen fixation and phytohormone production (Gardener, 2004). In industrial applications, their enzymatic activities are important for manufacturing processes in the detergent, paper and pharmaceutical sectors because of their ability to decompose complex organic materials (Grady et al., 2016). *Paenibacillus* spp. in food systems such as dairy products can have both beneficial and adverse effects. Some strains of some *Paenibacillus* spp. can cause food spoilage by producing undesirable flavour in dairy products but they can also provide potential benefits such as probiotic properties and the production of natural preservatives that could inhibit pathogenic micro-organisms (Spanu, 2016; Ranieri et al., 2009).

This literature review will provide a detailed scope on the nature of *Paenibacillus* spp. by exploring their applications and challenges in agricultural, industrial and food sectors with a focus on their impact in the dairy industry. The enzymatic activity and adaptive strategies of these bacteria were also examined with their effects in industry. The role of *Paenibacillus* spp.

is important and ongoing innovative studies are needed to optimize their beneficial uses while reducing the potential risks.

2.2 Overview and applications of *Paenibacillus* spp.

Paenibacillus spp. are rod-shaped, spore-forming, gram-positive bacteria and mesophilic (Blombach et al., 2022). The optimal growth conditions for *Paenibacillus* spp. are 28 to 40 °C at a neutral pH (Patowary & Deka, 2020). They can be discovered in environments such as soil, water, and plant roots. *Paenibacillus* spp. are facultative anaerobic or strictly aerobic (Grady et al., 2016) metabolic capabilities therefore they can grow in different environments.

Paenibacillus spp. have been studied across many areas including agriculture, industry, and environmental management due to its adaptability and enzymatic activity. *Paenibacillus* spp. can play the role of saprophytes that can decompose organic matter and promote nutrient cycling and increase the fertility of soil (Gardener, 2004). *Paenibacillus* spp. can also enhance plant growth through biological nitrogen fixation by converting nitrogen into a form usable by plants (Gardener, 2004). It is important to reduce the use of chemical fertilizers to promote sustainability in agriculture. However, *Paenibacillus* spp. can also cause problems in different areas. *Paenibacillus* spp. detected in food can cause spoilage in dairy products where they produce enzymes that degrade milk quality (Spanu, 2016; Ranieri et al., 2009). It is a challenge for the food industry, and it requires monitoring and control to decrease the risk of contamination and spoilage.

Paenibacillus spp. can produce enzymes including amylases, cellulases, hemicellulases, proteases and lipases that have various applications such as the manufacturing of detergents, paper production, and healthcare products (Grady et al., 2016). These enzymes can be used to break down complex molecules that improve the stain removal in detergents and the breakdown of biomass in production of paper. *Paenibacillus* spp. can also be used for bioremediation where biological agents degrade or detoxify pollutants from contaminated sites such as heavy metals (Govarthanan et al., 2016). Therefore, it is possible to use *Paenibacillus* spp. in mitigating environmental pollution by improving soil health and restoring ecosystems affected by industrial waste.

2.2.1 *Paenibacillus amylolyticus*

Paenibacillus amylolyticus is a facultative anaerobe, spore-forming and mesophilic that is distributed in environments including soil and organic material (Bacdive, 2023; Lincoln et al., 2019) and it has been studied within range of ecological diversity, pathogenic potential, and biotechnological applications according to recent studies.

Paenibacillus amylolyticus has a strong adaptability to different habitats (Lincoln et al., 2019) such as soil and it can play an important role in nutrient cycling and decomposition of organic matter. *Paenibacillus amylolyticus* can also adapt to hostile environments including industrial waste as it was found to be able to carry out biodegradation processes. For example, *Paenibacillus amylolyticus* can degrade polyvinyl alcohol in dyeing wastewater along with *Microbacterium barkeri* (Choi, 2004). However, the growth of *Paenibacillus amylolyticus* can also cause potential health risks. *Paenibacillus amylolyticus has been* reported to be isolated

from human blood in Spain as a pathogen that resists common antibiotics like Rifampicin and Cotrimoxazole (Sáez-Nieto et al., 2017). *Paenibacillus amylolyticus* can also infect animals such as dogs (Rampacci et al., 2022) and it showed resistance to clindamycin and imipenem. These findings introduced that *Paenibacillus amylolyticus* can cause infections in both humans and animals as a pathogen. Another case studied by Wenzler et al. (2015) indicated that *Paenibacillus amylolyticus* can cause severe sepsis stemming from environmental contamination which can cause critical health hazards, but such instances are rare. *Paenibacillus amylolyticus* can also contaminate dairy environments (Trmčić et al., 2015). Strains of *Paenibacillus amylolyticus* were extracted with other dairy spoilers that showed proteolytic and lipolytic activities. The enzymes produced by *Paenibacillus amylolyticus* can break down proteins and fats that affect the quality and shelf life of dairy products. Therefore, it is important to be studied for the dairy industry. *Paenibacillus amylolyticus* also has biotechnological applications. It can produce glucoamylase that was applied for the saccharification process in fermentation at 37°C (Lincoln et al., 2019). The enzyme activity of glucoamylase could also be used for improving the efficiency of starch-based industrial processes.

Paenibacillus amylolyticus has been studied in areas such as ecological, health, and industrial use. Its ability to produce enzymes shows great potential in industrial application. However, it can be a pathogen with antibiotic resistance traits (Sáez-Nieto et al., 2017; Rampacci et al., 2022; Wenzler et al., 2015) that need to be controlled and further studied.

2.2.2 *Paenibacillus polymyxa*

Paenibacillus polymyxa is a gram-positive, rod-shaped bacterium that is important in ecological and biotechnological areas. It can be found in soil, rhizosphere and the roots of crop plants that can impact the environment such as plant and soil health (He et al., 2007). *Paenibacillus polymyxa* can form endospores that can survive in harsh conditions, and it is a non-pathogenic bacterium, so it is safe for different applications (Timmusk et al., 2005).

Paenibacillus polymyxa can produce polymyxins as antibiotics used for gram-negative infections (Sánchez, 2011) and it is important in biotechnological use. It is important to decrease the adverse effects and make an improved use of the antibacterial properties for medical use. *Paenibacillus polymyxa* also has important applications in agriculture including plant growth promotion through the production of growth-stimulating hormones, antibiotics that protect plants from pathogens and nitrogen fixation that enriches soil fertility (Lal & Tabacchioni, 2009). These features of *Paenibacillus polymyxa* in agriculture can promote the development of sustainable farming processes where chemical uses are minimized. *Paenibacillus polymyxa* can also be applied due to its enzyme production ability as it can produce enzymes that carry out glucanolyticus, chitinolytic and proteolytic activities (Nielsen & Sørensen, 1997). These enzymes are crucial in various industrial processes including the degradation of organic material that can be applied in waste management and raw material processing in industries such as textiles and pharmaceuticals.

Paenibacillus polymyxa has various applications in environmental management, agriculture and medicine areas. Its role in promoting plant growth and protecting crops can promote sustainability agricultural processes. The enzymatic products by *Paenibacillus polymyxa*

provide benefits for various industrial applications that provide potential uses in both natural and industrial ecosystems. The clinical use of its antibiotic properties is important to be balanced with its toxicity that require further study and development in the use of products derived.

2.2.3 *Paenibacillus glucanolyticus*

Paenibacillus glucanolyticus was first isolated from soil in England and is known for its biochemical capabilities and environmental significance (Alexander & Priest, 1989). It is a gram-positive, rod-shaped bacterium and it can grow in an anaerobic environment. It has an optimal growth pH of 9 and a temperature of 37°C. *Paenibacillus glucanolyticus* has potential for use in bioremediation and industrial waste management in the pulp and paper industries for degrading waste which can reduce environmental impact (Mathews et al., 2014). *Paenibacillus glucanolyticus* can also degrade lignocellulose and lignin that is challenging to break down through chemical processes and is important to plant biomass in the ecological system. The potential use *Paenibacillus glucanolyticus* in bioremediation and industrial waste management in the pulp and paper industries degradation which can reduce environmental impact (Mathews et al., 2014)

Paenibacillus glucanolyticus was found to be potentially pathogenic and it was resistant to penicillin and has difficulty in treatment of infections caused by this bacterium (Celandroni et al., 2016). There were also cases of keratitis and corneal perforation in humans infected with *Paenibacillus glucanolyticus* that indicate its clinical risks and the need for medical awareness (Hassan et al., 2021). *Paenibacillus glucanolyticus* can also provide agricultural benefits

despite these pathogenic risks. It can increase potassium solubility that can enhance plant growth significantly (Sangeeth et al., 2012). This indicated that *Paenibacillus glucanolyticus* can be used as an agent and eco-friendly alternative to chemical fertilizers in agriculture by improving soil fertility and plant health.

Paenibacillus glucanolyticus is seen as a bacterium with applications in environmental management, industrial processes, and agriculture. It can break down lignocellulosic and lignin materials for the sustainable management of industrial waste and enhance soil and plant health to support sustainable agricultural processes.

However, it is considered an opportunistic potential pathogen in humans, and infections usually affect immunocompromised individuals (Grady et al., 2016). There are both challenges and opportunities for *Paenibacillus glucanolyticus* in biotechnology and microbial applications.

2.2.4 *Paenibacillus macerans*

Paenibacillus macerans has been characterized as a spore-forming, rod shape, gram-positive bacterium with a range of phenotype diversity (Department of Veterinary Disease Biology, 2011). It is also a bacterium that is important in ecological and industrial areas as it plays a crucial role in promoting plant growth through nitrogen fixation and grows in various environments from soil to the roots of plants. Nitrogen fixation can enrich soil fertility and reduce the need for chemical fertilizers and promote sustainable agricultural processes. Therefore, *Paenibacillus macerans* can be used in ecological balance and enhance agricultural productivity.

However, *Paenibacillus macerans* also has potential negative impacts in areas such as clinical and food production. *Paenibacillus macerans* can contaminate the environment and cause pseudobacteremia which bring the infected person into a condition where bacteria are present in blood samples but not infecting the body (Noskin et al., 2001). In rare cases, it can also infect healthy adults and exhibit resistance to common antibiotics such as erythromycin and penicillin that bring challenges in treatment (Szaniawski & Spivak, 2019). *Paenibacillus macerans* can have an impact in the food industry as the following study showed. It caused milk spoilage and produced odours that degrade milk quality (Olajide & LaPointe, 2020). This can affect the sensory attributes of dairy products and bring economic challenges for dairy producers.

On the other hand, *Paenibacillus macerans* has shown potential use in biotechnological areas. It can produce α-Cyclodextrin glycosyltransferase that is an enzyme used for the fermentation in the food industry (Li et al., 2014). The α-cyclodextrin glycosyltransferase can produce cyclodextrins that are used to encapsulate flavours and stabilize sensitive food components, so the quality and shelf life of foods are enhanced. *Paenibacillus macerans* can also produce histamine (Rodriguez-Jerez et al., 1994) for medical applications. Histamine can be used in clinic area including immune reactions and gastric acid secretion and *Paenibacillus macerans* can be a potential source for medical-grade histamine.

Paenibacillus macerans can be both beneficial and have negative impacts in different areas. It can support agricultural productivity and provide benefits through enzyme production, but its pathogenic and food spoilage potential require careful management.

2.3 Characterization of lipase and protease

Paenibacillus spp. can produce many different types of enzymes including proteases and lipases and these enzymes are important in different industries including bioremediation to pharmaceuticals and food production. These enzymes can be characterized using different methods and technologies. Spectrophotometry can be used to determine enzyme activity by light absorbance (Sharma et al., 2001). Chromatographic techniques like high-performance liquid chromatography (HPLC) and gas chromatography (GC) can be used for purifying enzymes and analysing their interactions with substrates (Bollag et al., 1996). Electrophoresis methods such as SDS-PAGE can also be used to estimate enzyme purity and activity and the mass spectrometry can provide precise molecular weight of enzymes (Smith et al., 2002).

2.3.1 Protease

Proteases are proteolytic enzymes that can act as biocatalysts in different biochemical processes. They can degrade proteins into smaller peptides or amino acids through proteolysis (Grady et al., 2016). There are four major classes for proteases based on the catalytic mechanism and residue involved in their catalytic activity including serine proteases, cysteine proteases, aspartic proteases, and metalloproteases (Rao et al., 1998). Proteases are used in different areas such as the dairy industry as they are commonly used for modifying milk proteins during cheese production. Proteases can be used for proteolysis by breaking down casein that is the main protein found in milk. It is important for cheese ripening as the proteases can hydrolyse casein into smaller peptides and free amino acids to modify different flavour, texture, and aroma of the cheese (Fox et al., 2004). The activity of proteases can increase the efficiency of the production process of cheese as the addition of proteases can

reduce the coagulation time of milk and enhance the yield and efficiency of cheese production. The breakdown of casein can also stabilize the micelles structure in milk for formation of curd (Fox et al., 2004). Proteases were also used in the leather industry to remove the hair on the leather and replace the chemical process for sustainability and leather quality (Thanikaivelan et al., 2004). Proteases were used as agents for treating wounds and burns and promote healing by removing necrotic tissue in pharmaceuticals (Powers et al., 2002). Proteases are also the key components in laundry and dishwasher detergents by breaking down protein-based stains to enhance cleaning efficiency (Maurer, 2004). Most of the thermostable proteins used in the industry are produced from thermophilic *Bacillus* sp. (Sinha & Khare, 2013).

2.3.2 Lipase

Lipases belong to a family of hydrolases that can catalyse the hydrolysis of ester bonds within lipid molecules. They can act as biocatalysts in the oil-water interface because it is non soluble in water and is used for insoluble substrates. Lipases can be produced from different sources including mammals, plants, and microorganisms. The microbial lipases were used the most in industrial applications due to their broader range of operational conditions and substrate specificities (Hasan et al., 2006). They are used in different industries including dairy as they can enhance flavours, modify fats, and improve shelf-life (Hasan et al., 2006). Lipases can also promote lipolysis by breaking down lipids in milk, so it is important in the dairy industry. The processes of lipolysis involve hydrolysis of triglycerides into glycerol and free fatty acids and is important in the production of cheese and butter for the modification and development of flavour and texture. For example, the breakdown of short and medium-chain fatty acids

through lipase activity can produce a distinctive flavour for certain cheeses such as blue cheese and Italian cheeses (Collins et al., 2003).

Proteases and lipases are important biocatalysts in the dairy industry by the proteolysis of milk proteins for cheese maturation and lipolysis for flavour and texture development in dairy products. The proteases and lipases produced by *Paenibacillus* spp. require further study for their industrial applicability to remain active and effective under thermal processing conditions and play an important role in biotechnological applications in different areas to expand the utility of lipases in industrial applications further.

2.3.3 Heat stability of lipase and protease

Some heat-stable enzymes can be produced by microorganisms according to previous studies. *Bacillus* spp. where *Paenibacillus* spp. originally belong to have been found to produce several heat-stable enzymes including amylases and cellulases (Hmidet et al., 2009). Heat-stable enzymes such as amylases are important as they can remain active and stable at high temperatures for processes like starch liquefaction that requires temperature above 80 °C (Horikoshi, 1999). These enzymes can break down starch into sugars at temperatures where many other microbial amylases would denature to increase the efficiency of the process. The heat-stable cellulases can be used for the biofuel industry by converting cellulosic biomass into fermentable sugars to produce bioethanol. It is important as these enzymes can operate at high temperatures and increase the speed of the hydrolysis of cellulose and reduce the risk of contamination (Viikari et al., 2007).

The heat stability of proteases is important to be studied. They can be influenced by their sources, structures, and the presences of stabilizing agents such as metal ions (Daroit et al., 2011).

Microbial proteases are more heat-stable compared to the proteases derived from plant or animal sources so they are widely used for industrial applications where processes might involve higher temperatures (Rao et al., 1998). The heat stability of proteases provides different applications in different areas such as the detergent industry with requirements to function in hot water for laundry processes. Heat-stable proteases can increase the cleaning ability of detergents by breaking down protein stains such as blood and food residues at washing temperatures at 60°C (Gupta et al., 2002). Heat stability of proteases has an important role in the dairy industry. It can be used to improve the solubility, digestibility, and bioactive properties of dairy products under thermal conditions (Kaur et al., 2024).

Heat stability of lipases is also studied indifferent areas in industry. Heat stability can keep the function of that lipases by maintaining their activity at high temperatures for controlled lipolysis. Some microbial lipases have been found to be able to remain activity after exposure to temperatures at 90°C so they are important to use in processes that require thermal resistance (Aravindan et al., 2007). The heat stability of lipases is also important for their application in industries requiring processes with high temperatures. The transesterification reaction to produce biodiesel from vegetable oils and animal fats also requires catalysts that are stable at high temperatures to ensure efficient conversion rates (Sharma et al., 2001). The use of heat-stable lipases can reduce the need for chemical catalysts and solve the environmental and safety concerns. Heat-stable lipases are also important in the dairy

industry because they can function in the pasteurization process without denaturing and catalyse fat hydrolysis after pasteurization. It can enhance the development of flavours in cheese (Collins et al., 2003) but they can also cause spoilage in other products.

The enzymes produced by *Paenibacillus* spp. function in high temperatures can be beneficial in industrial processes as they can operate under severe conditions. The risk of microbial contamination can be reduced for these enzymes when it is desirable, and the reaction rates will also increase. It can also extend the range of the applications for different industries such as biotechnology, pharmaceuticals and food processing processes involving thermal processes. Further study and development are required to exploit these properties involving the protein structure.

Some *Bacillus* isolates can also produce thermostable lipase functioning at over 60 °C (Hamdan et al., 2021). *Pseudomonas fluorescens* isolated from raw milk were tested to produce lipase that can be inactivated to 10 % after heat treat at 71.5 °C for 10 minutes and the lipase was also found to be less activate in skim milk than whole milk due to the activity of protease (Bucky et al., 1986). Three heat-stable proteases out of 17 proteases produced by *Pseudomonas* and *Moraxella* were treated for 15 s at 138°C and one heat-stable lipase was inactivated (Christen et al., 1986) indicating that the mechanism of inactivation of enzymes is different. The average inactivation percentage of lipases produced by psychrotrophic bacteria in raw milk were lower than 45% (Deeth, 2021) with different methods of heat treatment.

2.4 Spoilage and control in dairy industry

Paenibacillus spp. as facultative anaerobic and endospore-forming bacteria are important to be noticed in the dairy industry. *Paenibacillus* spp. can produce a wide range of enzymes and antimicrobial compounds that can influence milk processing and cheese production (Grady et al., 2016). Proteases and lipases are also the potential enzymes that *Paenibacillus* spp. can produce. These enzymes can break down proteins and fats to the release of peptides and free fatty acids that can affect the quality of dairy products. *Paenibacillus* isolates can also produce antimicrobial substances such as polymyxin, paenibacillin and other bacteriocins (He et al., 2007) and these compounds have potential applications as natural preservatives in dairy products to inhibit the growth of spoilage organisms and pathogens. These applications ensure the safety of dairy products and reduce the need for synthetic preservatives to meet the consumer demand for more natural products. *Paenibacillus* spp. have shown health benefits including enhancement of the immune response and improvement of gastrointestinal health for further development of functional dairy products (Gupta & Malik, 2007). However, it is important to note that the presence of *Paenibacillus* in dairy products is not always beneficial. Some isolates can cause spoilage and reduce shelf life in milk so the application of *Paenibacillus* spp. in the dairy industry requires careful strain selection and control (Fromm & Boor, 2004).

2.4.1 Spoilage potential in dairy industry

The spoilage activities of *Paenibacillus* spp. in the dairy industry is important to be studied. It can cause economic losses and affect the quality of the dairy products and survive pasteurization as they can form endospores that can bring challenges for dairy processes.

These spores can germinate when conditions are favourable after pasteurization to cause the spoilage of milk and dairy products. It includes various undesirable changes including change in flavours, sediment formation and changes in texture and appearance (Ivy et al., 2012).

Bacteria in dairy products have the potential ability to produce lipolytic and proteolytic enzymes. These enzymes can degrade lipids and proteins in the products to cause rancidity and bitterness in milk (Samaržija et al., 2012). Some psychrotrophic *Paenibacillus* isolates can cause spoilage potential in refrigerated products by producing heat-stable enzymes (Deeth, 2021). They can grow at low temperatures including most dairy storage conditions and cause spoilage during distribution and storage. The activity of these isolates in cold environments has been found to produce slime and other spoilage-associated compounds in milk and cheese (Te Giffel, 1996).

The heat-stable enzymes including protease and lipase are the main reasons for spoilage of UHT milk. Most of the protease produced in UHT milk are heat-stable and less than 50% of tested protease can be inactivated (Deeth, 2021). Protease from *Pseudomonas* has been found to be highly heat-stable that require the highest temperature of 160 °C for inactivation (Stoeckel et al., 2016). Furthermore, the thermal stable protease produced by *Bacillus* sp. is active at 75 °C to 85 °C (Sinha & Khare, 2013).

Lipase was found to causing spoilage in dairy products as well. Lipase in UHT milk can cause release of short chain or long chain fatty acid by lipolysis in UHT milk during storage at 23 °C and 35 °C and the spoilage would be more severe as storage time increases (Choi & Jeon, 1993). The lipase remained after heat treatment.

Paenibacillus spp. have been found to be one of the agents in the spoilage of cow milk. The flavour and viscosity were changed due to the enzyme activity (Clark, 2017). *Paenibacillus* isolates can also produce biofilms on dairy processing equipment. They can cause physical contaminants in the equipment and protect the bacteria from cleaning and sanitization processes so that they are harder to eliminate. The biofilms can cause persistent spoilage issues and cross-contamination that is harmful to keep the hygiene and safety of the production environment (Flint et al., 2001).

2.4.2 Control and Management in dairy industry

The control and management of *Paenibacillus* spp. in the dairy industry is important due to the potential of spoilage. The management strategies were studied to help reduce the risks and enhance the benefits for product development.

The common method for inactivation of bacteria and enzyme is heat treatment and the main aim of heat treatment is to minimize the spoilage by inactivating the enzymes to reduce chemical and physical changes (Tamime, 2009). The effect of heat treatment on enzymes can be different due to different bacteria isolates and different strains of same isolates (Deeth, 2021).

One method of heat treatment is thermisation that has a condition at 57 to 68 °C for 15 seconds to partially inactivate the enzymes and keep the quality of raw milk (Tamime, 2009).

This method has lower heat load that does not change the quality of milk (van den Oever et al., 2021).

Pasteurization is also a method of heat treatment and the traditional pasteurization condition for milk is 63 °C for 30 minutes. The newer method for pasteurization involves HTST (High temperature, short time) and the minimum requirements for HTST is 72 °C for 15 seconds (Tamime, 2009). This method will introduce more heat load and can lead to form protein complexes by bounding denatured whey protein and k-caseins on the surface of micelles (van den Oever et al., 2021). The shelf-life of New Zealand pasteurised milk is 14 - 21 days, 5 to 10 days in Europe and about 20 days in the USA. There is still a risk of coagulation and loss of quality due to raw milk quality or recontamination (Rauh & Xiao, 2022).

Sterilisation is also commonly used to inactivate heat resistant spores to extend shelf life. For UHT milk, the condition includes the temperature of 121 °C for 3 minutes, or equivalent e.g. 144 °C for 4 seconds (Tamime, 2009). The shelf life is 3 to 12 months for UHT milk with a risk of lipolysis to cause bitterness due to enzymes that remain active. Sterilized milk is processed under conditions at 121 °C for 20 minutes and highest heat load was detected in this method with a risk of overprocessing that changes quality of milk (van den Oever et al., 2021).

Other methods to control *Paenibacillus* spp. in dairy environments is to use hygiene and sanitation processes. It is important to clean and sanitize dairy processing equipment and storage areas regularly to eliminate spores that may survive pasteurization. The sanitizers such as peracetic acid and hydrogen peroxide that can be effective against bacterial spores (Sadiq et al., 2017). The air conditions in processing facilities are also important to prevent airborne

spores from contaminating the product. *Paenibacillus* spp*.* can grow at a wide range of temperatures, and it is important to be considered for the management. Refrigeration at temperatures below 7°C can slow down the growth of these bacteria but some psychrotrophic strains may still grow. It is important to keep refrigeration during storage and transportation for dairy products (Flint et al., 2001). Bacteriocins produced by lactic acid bacteria can also be used to control *Paenibacillus* spp*.* They can inhibit the growth of spoilage organisms without affecting the sensory qualities of the dairy product. Bacteriocin such as Nisin is used in the dairy industry and can be effective against different spoilage bacteria such as *Paenibacillus* (Cleveland et al., 2001). PCR and DNA sequencing are commonly used in identification of contamination sources. These techniques can reduce the time taken to respond to contamination and lower the impact on product quality and safety (Postollec et al., 2011). The change in pH and salt concentration in dairy products can also inhibit the growth of *Paenibacillus* spp. Lower pH levels and higher salt concentrations can reduce the viability of bacterial spores to decrease the chance of spoilage (De Giori et al.,1 985).

The management of *Paenibacillus* spp. in the dairy industry can be achieved by sanitation practices, temperature control, use of natural bacteriocins, detection methods and process optimization and it is important for quality issue in dairy.

2.5 Evaluation of sources

2.5.1 Enzymes produced by *Paenibacillus* spp.

The enzyme activity and production by *Paenibacillus* isolates were tested to have high or medium growth at 37 °C for 24 hours and the growth was medium or low for *Paenibacillus* at 21 °C for 24 hours (Trmčić et al., 2015). The results by Trmčić can be used as a reference to decide the growth temperature but the growth will be different as different strains were tested in this study.

There were 5 strains of *Paenibacillus* spp. tested positive for proteolytic activity and 3 *Paenibacillus* were tested positive for lipolytic activity out of 16 strains tested by Trmčić. The enzyme activity tested by Trmčić can also be a reference in this study for different strains of *Paenibacillus spp*. There were 16 strains of *Paenibacillus* studied by Trmčić and different isolates will be tested to further study the characterization of enzymes as comparison in this study.

Some isolates of *Paenibcillus* can produce enzymes except proteases. *Paenibacillus amylolyticus* can produce glucoamylase that is active at 37 °C (Lincoln et al., 2019). The study by Li et al. (2010) showed the α-Cyclodextrin glycosyltransferase produced by *Paenibacillus macerans* has an optimum reaction temperature at 45 °C. In the study by Nielsen and Sørensen (1997), *Paenibacillus polymyxa* was isolated from soil and the enzyme activity was only tested for polysaccharide hydrolases. The isolation of *Paenibacillus* from a single source can limit the range of isolates and the enzyme production of protease and lipase by *Paenibacillus* are also important to be studied in this study. The saccharification by *Paenibacillus* was not studied but it provided the active conditions for various enzymes produced by *Paenibacillus* that has been studied. The production of other types of enzymes should also be considered to reduce the influences on the results.

The heat stability of enzymes is always important to be studied. The alkaline protease produced by an alkalophilic *Bacillus* strain can be active from a range of 30 °C to 80 °C with pH of 10 and the amylose can be active from 30 °C to 100 °C (Hmidet et al., (2009). The alkaline proteases were also studied by Horikoshi (1999) and the alkaliphilic Bacillus sp. strain AH-101 can produce alkaline protein that is stable at 60 °C for 10 minutes. The previous studies showed conditions for the heat-stable proteins as references in this study. Some strains of *Paenibacillus* spp. are also alkaliphilic (Priest, 2015) so it is possible for them to produce heat-stable alkaline protease in this study.

2.2.2 Spoilage potential and management of enzymes

Paenibacillus isolates were isolated and identified by Fromm and Boor (2004) for 39% of the cause of spoilage in processed milk. The numbers of *Paenibacillus amylolyticus* increased and reached the peak on day 14 of the shelf life where the other bacteria decreased in numbers. The results indicated the spoilage potential *Paenibacillus* spp. and the requirement of management. *Paenibacillus amylolyticus* was the only *Paenibacillus* isolated by Fromm and Boor so the results can be bias. Different dairy products including milk, cream, butter, and cheese had defects such as off-flavour and gelation due to the spoilage caused by enzymes produced by psychrotrophic bacteria (Samaržija et al., 2012). *Paenibacillus* was not tested by Samaržija but similar defects might be observed for *Paenibacillus* in dairy products.

The spoilage potential for psychrotrophic bacteria including *Bacillus* and *Pseudomonas* were found to produce heat-stable enzymes in a previous study (Deeth. 2021). The highest

temperature for inactivation of protease from *Pseudomonas* is 160 °C (Stoeckel, M. et al., 2016). This indicates that temperatures higher than 100 °C might be needed to inactivate protease produced if any psychrotrophic *Paenibacillus* isolated in this study. Christen et al. (1986) provides more detailed tests on activity with the percentage of inactivation for bacteria tested at 138 °C after 15 second. It is a relatively old study that mainly focused on enzymes produced by psychrotrophic bacteria. In that study, 50 % of psychrotrophic bacteria isolated produced heat-stable lipase or protease (Christen et al., 1986) and for *Paenibacillus* the rate will be different. Some of the *Bacillus* isolates were also found to produce enzymes that can be inactivated at less than 100 °C (Sinha& Khare, 2013; Hamdan et al., 2021). It is also suggested that lipases produced by *Pseudomonas fluorescens* can be deactivated at 71.5 °C (Bucky et al., 1986). These results can be used to compare with the results of *Paenibacillus* isolates in this study.

2.6 Conclusion

Paenibacillus spp. has been reviewed in different areas in the literature above. *Paenibacillus* genus is a group of bacteria with a wide range of applications that are both beneficial and problematic. They can improve agricultural productivity through nitrogen fixation and soil health and the enzyme produced can be used for detergents. They also play important roles in agriculture enhancement, industrial enzyme production and environmental bioremediation. It is important for them to contribute to sustainable practices and promote environmentally friendly processes in industry. *Paenibacillus* also plays an important role in the dairy industry including flavour development, food safety and product enhancement through its enzymatic and antimicrobial activities. The fermentation processes can be further

studied to expand the use of *Paenibacillus* in creating high-quality, safe and functional dairy products.

Paenibacillus can be beneficial in specific dairy applications but cause spoilage, so they need careful control and management in the dairy industry. The challenges brought by *Paenibacillus* can only be solved by continuing advancements in microbial management including the development of more targeted approaches in microbial application and control. The genetic and metabolic aspects of *Paenibacillus* spp. may also need to improve the utilization strategies in industrial and agricultural settings. Future study can also focus on the probiotic potential of *Paenibacillus* spp. to enhance the nutritional value and health benefits of dairy products. It is important for innovations in food processing and preservation to reduce the use of chemical additives and enhance the natural quality of dairy products.

In conclusion, the *Paenibacillus* genus has values for study and application in different sectors. It is required to decrease the associated risks and maximize its beneficial impacts. The continued exploration of *Paenibacillus* spp. will provide more sustainable and efficient biotechnological applications to further develop in agriculture, industry, and public health.

3. Materials and methods

3.1 Isolate *Paenibacillus* spp. from raw milk

The milk powder and raw milk samples were provided for isolation of *Paenibacillus*. The isolation method involves using milk samples prepared by adding 4 g of milk powder into

distilled water to get a total volume of 40 mL. Then, the samples were heated at 80 °C and stored at 7 °C for 10 days. The previous processes were repeated on day 1, day 5 and day 10. After that, the samples were diluted by adding 1mL of milk into 9mL of 0.1% Millipore peptone water for a 10^{-1} dilution. The peptone water was prepared by adding 1g of peptone water powder into 1L distilled water and were mixed and autoclaved at 121°C for 15 minutes. These processes were repeated twice to get 10^{-2} and 10^{-3} dilution. Milk samples were added with Oxoid Milk Plate Count Agar (MPCA) and were incubated at 37 °C for 24 hours. MPCA was prepared by adding 19.5g of MPCA powder into 1L distilled water and they were boiled and mixed in pot to dissolve. The mixture of MPCA were autoclaved at 121°C for 15 minutes. The individual bacteria colonies were isolated and streaked on MPCA for purity. The streaked plates were incubated at 37 °C for 48 hours.

In the end, the isolated colonies were picked and smeared onto a glass microscopy slides for gram staining. Crystal violet was used to stain the slide for one minute. Iodine solution was put on the slide to fix the stain after washing with distilled water. Ethanol was used to wash off the colour and then wash with distilled water. The slide was stained with safranin in the end for 30 seconds and washed off with distilled water. The slides were observed under light microscope (Olympus BH2-RFCA) with immersion oil at 1000x magnification to screen out gram-positive, rod shape bacteria. The isolated bacteria with gram-positive and rod shapes were streaked on Difco™ PCA plates to send for MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) mass spectrometry test analysis. *Paenibacillus* spp. used in this study were listed in Figure C1.

3.2 Detection of enzyme activities by Difco^{TM} PCA and skim milk powder

The agar slants were prepared by boiling Difco^{TM} TSA power and water with ratio according to the instruction and distributed in glass tubes. The tubes were autoclaved at 121°C for 15 minutes before use. The isolated *Paenibacillus* isolates were inoculated in agar slants and incubated at 37 °C for 24 hours (Trmčić et al., 2015). The enzyme activities of *Paenibacillus* isolates were determined by milk agar plates and tributyrin agar plates. The milk agar was prepared by boiling Difco^{TM} PCA powder and water with 6% skim milk powder. The tributyrin agar was provided by Fort Richard laboratories (New Zealand). Both agars were autoclaved before use. The isolated isolates from agar slants were inoculated in prepared agar plates in triplicates and were incubated at 37 °C for 48 hours (Trmčić et al., 2015). The clear zone on plates was used as indication for positive (+), strong positive (++) or negative (-) results of protease and lipase and the results were compared with Bhakthavalsalam et al. (2018). For each isolate, a test of triplicate was repeated until a consistent result of positive or negative (all three plates shew positive, strong positive or negative) was acquired and taken as a valid result (See Figure B1, B2 and B3).

3.3 Temperature, medium and inoculum condition effects

The temperature effects were tested by growing selected *Paenibacillus* strains from agar slant at 37 °C and 30 °C in TSA plates. Each strain was streaked onto Difco^{TM} PCA plates with skim milk and tributyrin agar plates were incubated at 30°C and 37°C for 48 hours. The negative

controls were prepared for each temperature. The sizes of the clear zones were compared between the plates growing in different temperatures to determine the temperature effect on enzyme production.

Full strength TSB, 10% TSB and 1% skim milk broth were prepared for selected *Paenibacillus* isolates to test the effect of growth medium on enzyme producing ability. Full strength TSB was used as a control, 10% TSB was used to see the effect of reduced strength on ability of hydrolysis and 1% skim milk was used as a comparison with compositions in the medium. The composition of TSB included pancreatic digest of casein and papain digest of soybean whereas skim milk contained protein. Full strength TSB broth was made by mixing Difco™ TSB powder and water with ratio according to the instruction. The 10% TSB was prepared by diluting TSB water and the skim milk was prepared by dissolving 10 grams of skim milk powder into 1000 mL distilled water and were then autoclaved at 100 °C. The selected *Paenibacillus* isolates from agar slants were streaked on TSA plates for 24 hours at 37 °C. The bacteria from TSA plates were then incubated in 10 mL of 3 different medium at 37 °C for 24 hours and streaked to PCA plates with skim milk and tributyrin agar plates. The negative controls were prepared for 3 different mediums. The plates were then incubated at 37 °C for 48 hours to test for protease and lipase hydrolysis. The sizes of the clear zones were compared between the plates from different medium and bacteria from TSA for each strain to determine the medium effect on enzyme production.

The effects of the inoculum conditions (fresh and chilled) were determined by comparing the ability of enzyme production for selected *Paenibacillus* isolates in different conditions. The first group of *Paenibacillus* strains were incubated in agar slants for 24 hours at 37 °C (Trmčić et al., 2015) and were then stored at 4 °C for three weeks and there was no growth at 4°C

(Blombach et al., 2022). The second group of *Paenibacillus* strains were streaked on TSA plates and incubated for 24 hours at 37 °C for comparison. Both groups were streaked on PCA plates with skim milk and tributyrin agar plates and they were incubated at 37 °C for 48 hours (Trmčić et al., 2015). The negative controls were prepared for each inoculum conditions (fresh and chilled). The sizes of the clear zones were compared for each strain to determine the effect of inoculum conditions (fresh and chilled) on enzyme production.

3.4 Enzyme activities and Heat stability

The centrifuge tubes and TSB solution were prepared for selected *Paenibacillus*. TSB medium is used to ensure nutrient needed for the growth of bacteria. It also outcompetes milk medium on the general purpose of use. The selected *Paenibacillus* isolates from agar slants were streaked on TSA plates in triplicates for one time 24 hours at 37 °C for one time. Single isolated colonies of *Paenibacillus* spp. were picked and added into 10 mL TSB broth solution in 15 mL centrifuge tubes. TSB solutions were incubated at 37 °C for 48h hours (Trmčić et al., 2015). Negative controls were prepared without *Paenibacillus* isolates. Incubated tubes were centrifuged at 4400 *g* for 10 minutes. Two ml of supernatant was filtered with 0.2 µm film. The filtered solution was divided into 2 groups, one is heated in a water bath for 10 min at 100 °C to inactivate any enzymes in it (Tamime, 2009; Sinha& Khare, 2013; Hamdan et al., 2021) and the other group was untreated as control. The plates were prepared using same methods for skim milk and tributyrin agar from Fort Richard laboratories and a hole was drilled in the middle of each plate with a diameter of 0.5 cm. An amount of 50 µL of supernatant from each

group were transferred in the hole for each plate, the plates were then incubated at 37 °C for 48 hours and clear zone around the hole were observed and recorded.

The measuring of enzyme activities of *Paenibacillus* by acocasein assays and p-nitrophenol palmitate assays were made using methods by Teh et al. (2013) and Teh et al. (2012). The centrifuge tubes (15 mL) Falcon and TSB broth were prepared using same method for selected *Paenibacillus* isolates. The incubated TSB tubes were centrifuged at 4400 *g* for 10 minutes at room temperature before taking the supernatant. Two mL of the centrifuged supernatant was taken and divided into 2 groups, one is heated in a water bath for 10 min at 100 °C to inactivate any heat-stable enzymes in it (Tamime, 2009; Sinha& Khare, 2013; Hamdan et al., 2021) and the other group was untreated as control.

Three percent azocasein solution was prepared by mixing azocasein, 5mM phosphate buffer, 0.1% sodium azide and 0.1 mg/mL chloramphenicol. Sodium azide and chloramphenicol were added for prevention of bacteria growth during incubation. An amount of 0.1 mL supernatant was added in 0.9 mL 3% azocasein solution for both groups. The mixtures were incubated at 37℃ for 24h. Twenty percent trichloroacetic acid was prepared to stop the reaction. An amount of 0.4 mL azocasein solution was added with 0.8 mL 20% trichloroacetic in tubes for centrifugation. The tubes were then centrifugated at 4400 *g* for 10 minutes. The supernatant of 0.2 mL was transferred in a 96 well microtitre plate in triplicates under absorbance reading at 400 nm for three times. One unit of proteolysis by protease was defined as 1 µL of azocasein reacted with the process above in 1 mL solution (Bussamara et al., 2010; Teh et al., 2012).

The method used to prepare enzymes solutions for lipolysis was the same as protease which

includes incubation in TSB broth and centrifugation at 4400 *g* for 10 minutes at room temperature. An amount of 0.6 mL of the incubated PPNP solution were prepared by dissolving 0.5g p-nitrophenol palmitate, 4 g Triton X-100, 0.2 mg arabic gum, 0.1 g chloramphenicol and 0.1% sodium azide in 100 mL isopropanol and 900 mL of 50 mM Tris-HCl solutions in pH 8. The supernatant was taken 0.1 mL to incubate with 0.9 mL PPNP solution at 37°C for 8 hours. 0.6 mL of the incubated solutions were mixed with 0.6 mL of 96% ethanol to stop the reaction after incubation. The mixtures were centrifugated at 4400 rpm for 10 minutes to get supernatant. The supernatant of 0.2 mL was transferred in a 96 well microtitre plate in triplicates under absorbance reading at 405 nm for three times. One unit of lipolysis was defined as 1 µmol of p-nitrophenol palmitate reacted with the process above in 1 mL solution (Bussamara et al., 2010; Teh et al., 2013).

4. Results

4.1 Isolation results

There were 10 bacteria isolated from raw milk and milk powder. Two isolates were observed to be rod shape and gram-positive under microscope shown in Figure 1 and 2. The isolated isolates were sent for MALDI-TOF analysis for identification and the results were *Bacillus licheniformis* and *Bacillus cereus*.

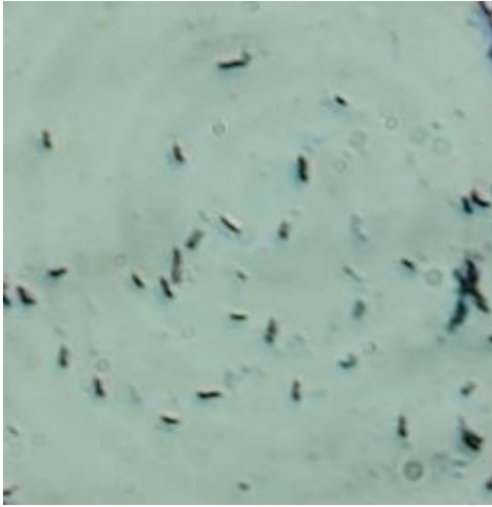

Figure 1: Bacteria 1 extracted under microscope under magnifications of under 10x100 magnification.

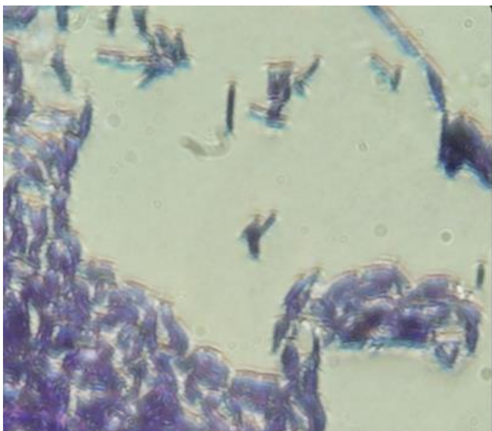

Figure 2: Bacteria 4 extracted under microscope under magnifications under 10x100 magnification.

4.2 Agar plates results and temperature effect

There were pre-isolated *Paenibacillus* species from raw milk in Massey University microbiology lab were used for proteolysis and lipolysis (See Table C1) and the results are

shown in Table 1 below. Seven isolates were found to be strong positive for proteolysis at 37 °C. Two isolates were found to be positive for proteolysis at 30 °C and the other two isolates were found to be strongly positive for proteolysis at 30 °C. No isolates were found to be positive or strong positive for lipolysis at 30 and 37 °C (see Figure B1 and B2 for pictures of positive plates).

Table 1: Results of proteolysis and Lipolysis of *Paenibacillus* isolates in different temperature of growth. For each isolates the experiment was carried out in triplicate with same results. Strong positive (++) is decided by the scale of clear zone (see Figure B2).

Label	Bacteria ID	Proteolysis at 30°C	Proteolysis at 37°C	Lipolysis at 30°C	Lipolysis at 37°C
s73	*Paenibacillus amylolyticus*	-	-	-	-
s630	*Paenibacillus amylolyticus*	-	-	-	-
w317	*Paenibacillus amylolyticus*	-	-	-	-

g54	*Paenibacillus amylolyticus*	-	-	-	-
w321	*Paenibacillus amylolyticus*	-	-	-	-
t437	*Paenibacillus amylolyticus*	-	-	-	-
w320	*Paenibacillus amylolyticus*	-	-	-	-
t84	*Paenibacillus amylolyticus*	-	-	-	-
t311	*Paenibacillus amylolyticus*	-	-	-	-
t348	*Paenibacillus amylolyticus*	-	-	-	-
t365	*Paenibacillus amylolyticus*	-	-	-	-

a337	*Paenibacillus macerans*	+	++	-	-
w295	*Paenibacillus macerans*	-	-	-	-
t440	*Paenibacillus macerans*	-	-	-	-
t485	*Paenibacillus macerans*	-	-	-	-
t85	*Paenibacillus macerans*	-	-	-	-
t82	*Paenibacillus macerans*	-	-	-	-
t70	*Paenibacillus macerans*	-	-	-	-
a517	*Paenibacillus glucanolyticus*	++	++	-	-

w284	*Paenibacillus glucanolyticus*	++	++	-	-
w82	*Paenibacillus glucanolyticus*	-	-	-	-
a505	*Paenibacillus polymyxa*	-	++	-	-
w84	*Paenibacillus polymyxa*	+	++	-	-
t418	*Paenibacillus polymyxa*	-	-	-	-
w280	*Paenibacillus polymyxa*	-	++	-	-
t322	*Paenibacillus polymyxa*	-	++	-	-

4.3 Inoculum conditions and medium effects

The results for effect of Inoculum conditions (fresh and chilled) are shown in Table 2 below. There were no obvious differences for proteolysis between day 1 (fresh) and day 31 (chilled).

Table 2: Results of Inoculum conditions (fresh and chilled) effects of *Paenibacillus* isolates in different day of growth. Strong positive (++) is decided by the scale of clear zone (see Figure B2).

Label	Bacteria ID	Amount of Proteolysis on	
		Day 1	Day 31
a337	*Paenibacillus macerans*	++	++
a517	*Paenibacillus glucanolyticus*	++	++
w284	*Paenibacillus glucanolyticus*	++	++

a505	*Paenibacillus polymyxa*	++	++
w84	*Paenibacillus polymyxa*	++	++
w280	*Paenibacillus polymyxa*	++	++
t322	*Paenibacillus polymyxa*	++	++

The results for the effects of proteolysis of 7 *Paenibacillus* isolates grown in different mediums are shown in Table 3 below. All 7 isolates (a337, a517, w284, a505, w84, w280 and t322) produced strong proteolysis in full strength TSB. Meanwhile, 6 isolates (a337, a517, a505, w84, w280 and t322) produced weak proteolysis in 10% TSB. Three isolates (a517, w284 and t322) produced strong proteolysis in 1% skim milk broth. Four isolates produced weak proteolysis in 1% skim milk broth.

Table 3: Results of medium effects of *Paenibacillus* isolates in different temperature of growth. Strong positive (++) is decided by the scale of clear zone (see Figure B2).

Label	Bacteria ID	Proteolysis in		
		TSB	10% TSB	1% Skim Milk
a337	*Paenibacillus macerans*	++	+	+
a517	*Paenibacillus glucanolyticus*	++	+	++
w284	*Paenibacillus glucanolyticus*	++	++	++
a505	*Paenibacillus polymyxa*	++	+	+

w84	*Paenibacillus polymyxa*	++	+	+
w280	*Paenibacillus polymyxa*	++	+	+
t322	*Paenibacillus polymyxa*	++	+	++

4.4 Enzyme activity and Heat stability results

The results for isolates with enzyme production are shown in Table 4 and Figure 3. *Paenibacillus amylolyticus* (w284) produced the highest amount of proteolysis amount of proteolysis of 72 U/mL. There were 6 isolates had a range of 5 to 9 U/mL for proteolysis. No isolates were found to produce obvious amount of lipolysis as all isolates had amount less than 1 U/mL. No obvious amount of proteolysis and lipolysis were found after heat treatment at 100 °C for 10 minutes as all results were less than 1 U/mL. Seven isolates of *Paenibacillus* were found to be significant different in means of proteolysis of enzyme (see Figure C1 and C2).

Table 4: For each isolates the tests were repeated in triplicate to provide mean value with standard deviations. The letters represent the significant difference in proteolysis or lipolysis with P < 0.05 (Turkey's test). The values after ± are standard deviations of the hydrolysis.

Label	Bacteria ID	Proteolysis (U/mL)		Lipolysis (U/mL)	
		No heating	Heated at 100°C	No heating	Heated at 100°C
a337	*Paenibacillus macerans*	5.091 ± 1.276[a]	0.111 ± 0.005[b]	0.629 ± 0.011[a]	0.587 ± 0.014[b]
a517	*Paenibacillus glucanolyticus*	9.106 ± 8.422[a]	0.115 ± 0.11[a]	0.622 ± 0.006[a]	0.588 ± 0.011[b]
w284	*Paenibacillus glucanolyticus*	72.3 ± 30[a]	0.120 ± 0.005[b]	0.630 ± 0.008[a]	0.592 ± 0.032[a]
a505	*Paenibacillus polymyxa*	5.911 ± 1.354[a]	0.154 ± 0.072[b]	0.634 ± 0.021[a]	0.589 ± 0.006[b]
w84	*Paenibacillus polymyxa*	5.558 ± 0.871[a]	0.107 ± 0.012[b]	0.637 ± 0.032[a]	0.599 ± 0.051[a]
w280	*Paenibacillus polymyxa*	6.089 ± 1.12[a]	0.157 ± 0.518[b]	0.593 ± 0.044[a]	0.572 ± 0.33[a]
t322	*Paenibacillus polymyxa*	6.708 ± 1.617[a]	0.110 ± 0.008[b]	0.646 ± 0.018[a]	0.621 ± 0.028[a]

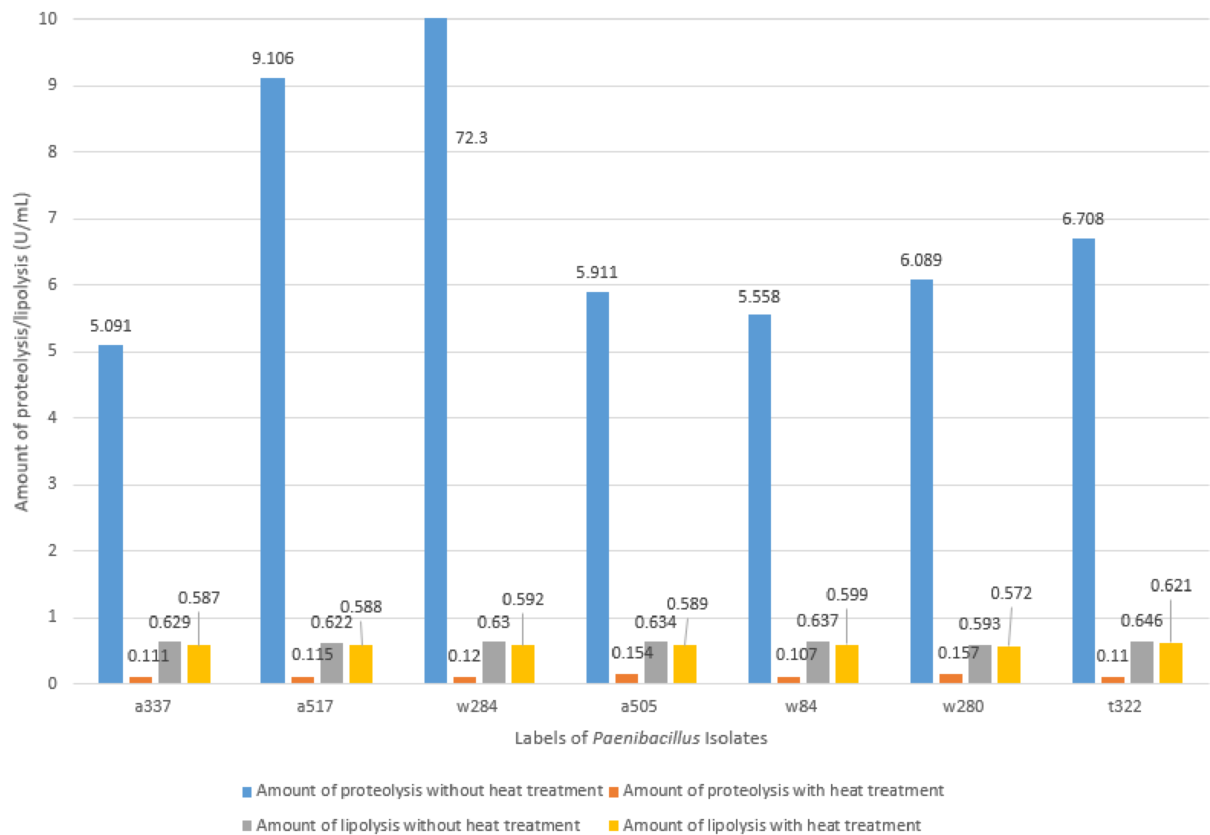

Figure 3: Amount of proteolysis and lipolysis with or without heat treatment.

5. Discussion

Paenibacillus isolates are rod-shaped, spore-forming, gram-positive bacteria (Blombach et al., 2022) and isolates from raw milk meet the criteria. The results by MALDI-TOF indicated that none of these isolates are *Paenibacillus* and future studies are required for more isolations.

Table 1 shows strain differences in amounts of enzymes activity under different temperatures. This is in accordance with results found by Trmčić et al. (2015). Some studies have found that the optimum growth temperature range of between 30 to 37°C is also optimum for enzyme production for a variety of mesophilic bacteria, including *Paenibacillus* (Blombach et al., 2022).

Table 3 shows strain differences in amounts of proteolysis in different mediums. This is also shown in a study by Bendicho et al. (2002). The amounts of proteolytic activity in whole milk, skim milk and ultrafiltrate skim milk are different. Future studies are required for different proteases produced when different nutrients were used. Table 2 shows no difference in terms of proteolytic activities on inoculum conditions. The proteolysis in 10% TSB and 1% skim milk suggests that *Paenibacillus* the strength of TSB can influence amount of proteolysis and higher protein content do not affect proteolysis.

Table 4 indicates that the enzyme activities were inactivated after heat treatment and no heat-stable enzymes remained active. Other literatures have found that the proteolytic activities were inactive after heat treatment under 55 °C and 60 °C for 20 and 10 minutes (Hang et al., 2016). It agrees with this study in which *Paenibacillus* was heated at 100 °C for 10 minutes.

6.Conclusion

The results of this study have expanded the production of enzymes by *Paenibacillus* as well as the characterizations of the enzymes. There were no isolates found to produce lipolysis and 7 isolates were found to produce proteolysis, and these results provides new insight on *Paenibacillus* in dairy products. The incubation temperature (30 and 37 °C) and inoculum condition (fresh and chilled) had no influences on the proteolytic activities. Different temperatures of incubation should be investigated in future study. All 7 isolates produce less proteolysis growing in 10% TSB and 4 out of 7 isolates produce less proteolysis in 1% skim milk and more studies are needed to investigate the medium effects on enzyme production. There

was no hydrolysis produced after heating at 100 °C for 10 minutes for enzymes produced by *Paenibacillus* isolates so no production of heat-stable enzymes was discovered. For future study different heating method and temperature need to be tested to expand the topic.

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Appendices

Appendix A: Materials and Equipment used in this work

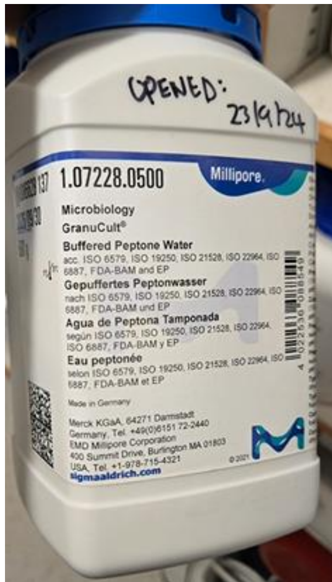


Figure A1: Millipore Peptone water used in methods.

Figure A2: Difco^{TM} TSA used in methods.

Figure A3: Oxoid Milk plate count agar used in method.

Figure A4: Difco™ PCA used in method.

Figure A5: Difco^{TM} TSB used in method.

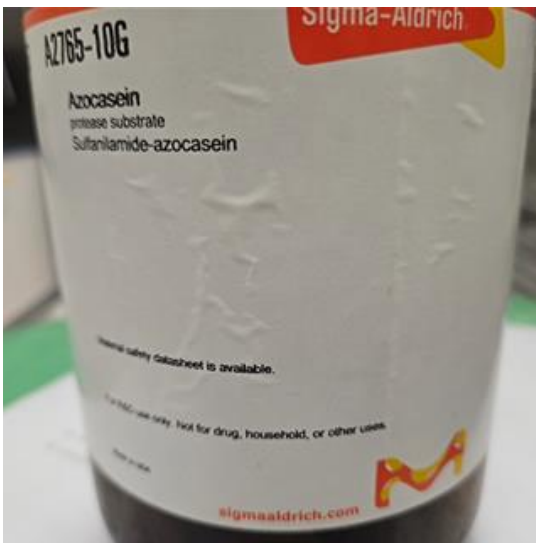


Figure A6: Sigma-Aldrich Azocasein used in methods.

Figure A7: Sigma-Aldrich Chloramphenicol used in methods.

Figure A8: Tributyrin agar from Fort Richard laboratories used in methods.

Figure A9: Olympus BH2-RFCA microscope used in methods.

Figure A10: BMG LABTECH SPECTROstar Nano Microplate Reader used in methods.

Figure A11: Eppendorf Centrifuge 5702 used in methods.

Figure A12: Falcon 96 well microtitre plate used in methods.

Figure A13: Skim milk powder used in the methods.

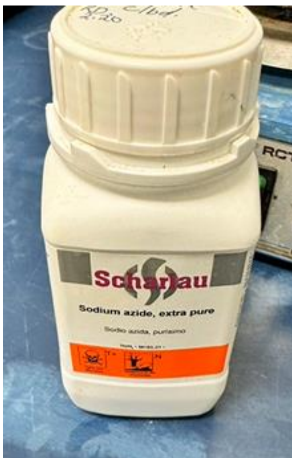


Figure A14: Sodium azide used in the methods.

Figure A15: 4-Nitrophenyl palmitate used in the methods.

Appendix B: Pictures of plates

Figure B1: Example of a plate with positive result (+) in proteolysis. Positive result shows half transparent area around the edge of growth area in medium ratio. The results were compared with Bhakthavalsalam et al. (2018).

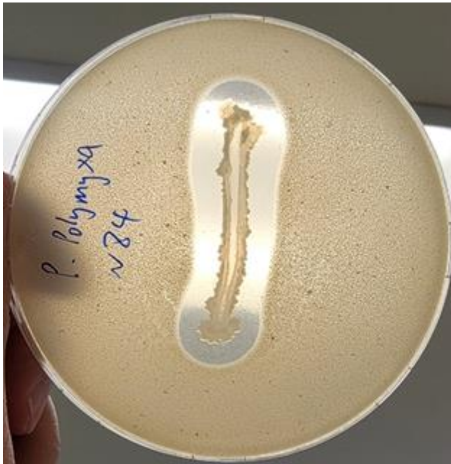


Figure B2: Example of a plate with strong positive result (++) in proteolysis. Strong positive result shows full transparent area around the edge of growth area in large ratio. The results were compared with Bhakthavalsalam et al. (2018).

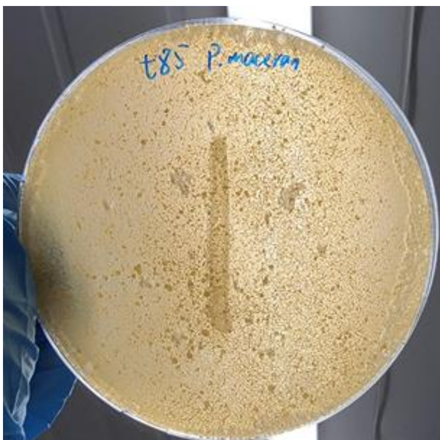


Figure B3: Example of a plate with negative result (-) in proteolysis. Negative result shows no transparent area around the edge of growth area. The results were compared with Bhakthavalsalam et al. (2018).

Appendix C: Raw data and calculations.

Table C1: List of *Paenibacillus* isolates used in this study.

No	Label	Season	Bacteria ID	Isolated from	Location
1	s73	Summer	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	s630	Summer	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	w317	Winter	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	g54	Spring	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	w321	Unknown	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	t437	Unknown	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	w320	Unknown	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	t84	Unknown	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	t311	Unknown	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	t348	Unknown	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	t365	Unknown	*Paenibacillus amylolyticus*	Raw milk	Microbiology lab
1	a337	Autumn	*Paenibacillus macerans*	Raw milk	Microbiology lab
1	w295	Winter	*Paenibacillus macerans*	Raw milk	Microbiology lab
1	t440	Unknown	*Paenibacillus macerans*	Raw milk	Microbiology lab
1	t485	Unknown	*Paenibacillus macerans*	Raw milk	Microbiology lab
1	t85	Unknown	*Paenibacillus macerans*	Raw milk	Microbiology lab
1	t82	Unknown	*Paenibacillus macerans*	Raw milk	Microbiology lab
1	t70	Unknown	*Paenibacillus macerans*	Raw milk	Microbiology lab
1	a517	Autumn	*Paenibacillus glucanolyticus*	Raw milk	Microbiology lab
1	w284	Winter	*Paenibacillus glucanolyticus*	Raw milk	Microbiology lab
1	w82	Unknown	*Paenibacillus glucanolyticus*	Raw milk	Microbiology lab
1	a505	Autumn	*Paenibacillus polymyxa*	Raw milk	Microbiology lab

1	w84	Winter	*Paenibacillus polymyxa*	Raw milk	Microbiology lab
1	t418	Unknown	*Paenibacillus polymyxa*	Raw milk	Microbiology lab
1	w280	Unknown	*Paenibacillus polymyxa*	Raw milk	Microbiology lab
1	t322	Unknown	*Paenibacillus polymyxa*	Raw milk	Microbiology lab

Table C2: Raw data of absorbance measured.

Azocasein no heating

a337	a517	w284	a505	w84	w280	t322	t440	t311	t84	s73	w317	g54	t348	t82	t70	w295	T418	t365	w82	t85	s630
0.148	0.176	0.199	0.148	0.148	0.147	0.151	0.073	0.047	0.071	0.046	0.07	0.058	0.046	1.405	0.047	0.036	0.043	0.046	0.041	0.06	0.045
0.137	0.145	0.211	0.149	0.141	0.15	0.153	0.081	0.049	0.07	0.073	0.04	0.088	0.06	0.047	0.027	0.044	0.05	0.055	0.042	0.051	0.04
0.137	0.125	0.222	0.137	0.141	0.14	0.14	0.086	0.045	0.073	0.072	0.04	0.077	0.051	0.036	0.028	0.039	0.11	0.06	0.026	0.03	0.036

Azocasein heated at 100°C

a337	a517	w284	a505	w84	w280	t322	t440	t311	t84	s73	w317	g54	t348	t82	t70	w295	T418	t365	w82	t85	s630
0.038	0.037	0.04	0.059	0.037	0.099	0.039	0.034	0.03	0.027	0.03	0.027	0.024	0.022	0.043	0.033	0.028	0.031	0.027	0.031	0.03	0.025
0.04	0.04	0.042	0.039	0.041	0.047	0.04	0.03	0.025	0.032	0.03	0.025	0.026	0.024	0.046	0.029	0.028	0.036	0.029	0.031	0.031	0.027
0.038	0.042	0.04	0.039	0.035	0.049	0.036	0.034	0.024	0.023	0.027	0.028	0.035	0.024	0.041	0.025	0.029	0.03	0.028	0.034	0.03	0.029

PPNP no heating

a337	a517	w284	a505	w84	w280	t322	t440	t311	t84	s73	w317	g54	t348	t82	t70	w295	T418	t365	w82	t85	s630
0.05	0.05	0.054	0.047	0.042	0.005	0.057	0.019	0.028	0.009	0.028	0.014	0.037	0.032	0.051	0.048	0.064	0.064	0.042	0.07	0.051	0.065
0.063	0.054	0.063	0.071	0.064	0.043	0.068	0.03	0.051	0.014	0.065	0.021	0.068	0.024	0.048	0.062	0.059	0.059	0.091	0.046	0.055	0.051
0.059	0.057	0.057	0.063	0.079	0.058	0.077	0.022	0.002	0.019	-0.009	-0.028	0.026	-0.018	0.064	0.042	0.07	0.07	0.071	0.054	0.052	0.083

PPNP heated at 100°C

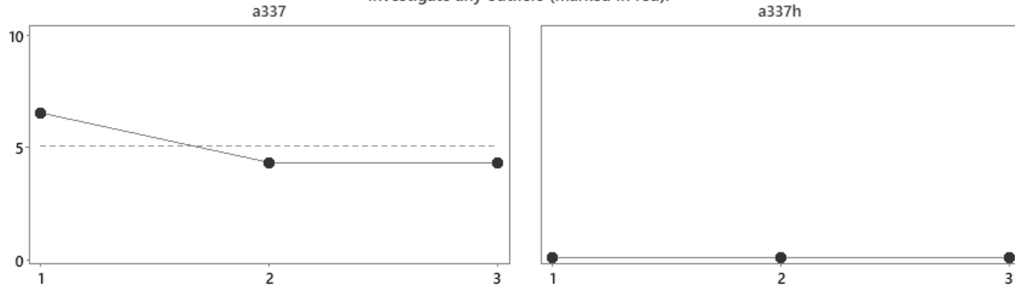
a337	a517	w284	a505	w84	w280	t322	t440	t311	t84	s73	w317	g54	t348	t82	t70	w295	T418	t365	w82	t85	s630
0.026	0.028	0.012	0.029	0.002	0	0.034	0.002	0.003	0.014	0.017	-0.004	0.008	0.001	0.05	0.051	0.046	0.033	0.039	0.018	0.032	0.023
0.028	0.035	0.045	0.036	0.058	0.025	0.065	0.01	0.004	0.023	0.03	0.015	0.015	0.057	0.028	0.058	0.057	0.059	0.059	0.039	0.038	0.042
0.042	0.036	0.048	0.035	0.056	0.042	0.059	0.014	-0.003	1.018	0.03	0.017	0.016	-0.03	0.056	0.053	0.066	0.056	0.076	-0.168	0.031	-0.008

Table C3: Mean amount of proteolysis and lipolysis. A triplicate of analysis was carried out for each mean value.

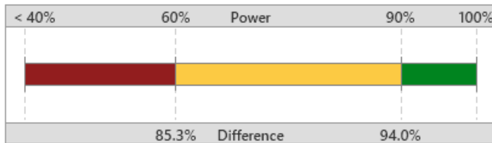
	Proteolysis（U/mL）		Lipolysis （U/mL）	
ID	no heating	heated at 100°C	no heating	heated at 100°C
a337	5.090865	0.110996743	0.6288165	0.586531195
a517	9.105592	0.115482533	0.6224551	0.588061906
w284	72.28847	0.119599046	0.6299339	0.59186738
a505	5.910838	0.153853502	0.634195	0.588594518
w84	5.558226	0.107332855	0.636841	0.598769178
w280	6.089001	0.157261766	0.5929247	0.571669474
t322	6.707729	0.109774103	0.6464473	0.621146024
t440	0.529021	0.088888123	0.5731739	0.550009187
t311	0.151676	0.070348586	0.5792831	0.542196179
t84	0.375836	0.073359415	0.5581214	0.56508156
s73	0.310085	0.077437607	0.6110745	0.576370805
w317	0.196792	0.070958091	0.563512	0.551152694
g54	0.463802	0.07668233	0.6062762	0.556579615
t348	0.189186	0.062628468	0.5800356	0.583324676
t82	0.125907	0.132423151	0.6236927	0.607544419
t70	0.099221	0.077906752	0.6174844	0.623027297
w295	0.116031	0.075442553	0.6410209	0.627177508
T418	0.629746	0.088005093	0.6410209	0.615371265
t365	0.198619	0.074532828	0.6484581	0.630444962
w82	0.105261	0.086615014	0.6278315	0.581041163
t85	0.167327	0.081289388	0.6207325	0.589134605
s630	0.119171	0.071902411	0.6449276	0.587426033

2-Sample Standard Deviation Test for a337 and a337h

Diagnostic Report



What is the chance of detecting a difference?



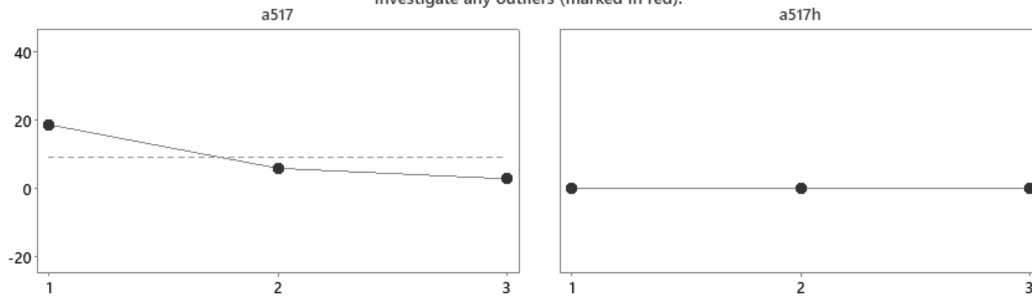
For α = 0.05 and sample sizes = 3:
If the true standard deviation of one sample were 85.3% smaller than the other, you would have a 60% chance of detecting the difference. If one were 94.0% smaller than the other, you would have a 90% chance.

What difference can you detect with your sample sizes of 3?

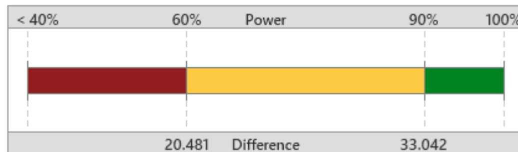
% Difference	Power
85.3%	60%
88.4%	70%
91.2%	80%
94.0%	90%

2-Sample t Test for the Mean of a517 and a517h

Diagnostic Report



What is the chance of detecting a difference?



For α = 0.05 and sample sizes = 3:
If the true means differed by 20.481, you would have a 60% chance of detecting the difference. If they differed by 33.042, you would have a 90% chance.

What difference can you detect with your sample sizes of 3?

Difference	Power
20.481	60%
23.643	70%
27.489	80%
33.042	90%

Observed difference = 8.9901

Figure C1: Example of comparison of mean and standard deviation in Minitab. For each mean value, a 2-Sample t Test was carried out to compare the difference between amount of hydrolysis with and without heat treatment. The results shew significant difference with $p \leq 0.05$ for 7 tested isolates.

One-way ANOVA: a337, a337h

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	2	a337, a337h

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	1	37.199	37.1986	45.68	0.002
Error	4	3.257	0.8142		
Total	5	40.456			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.902355	91.95%	89.94%	81.89%

Means

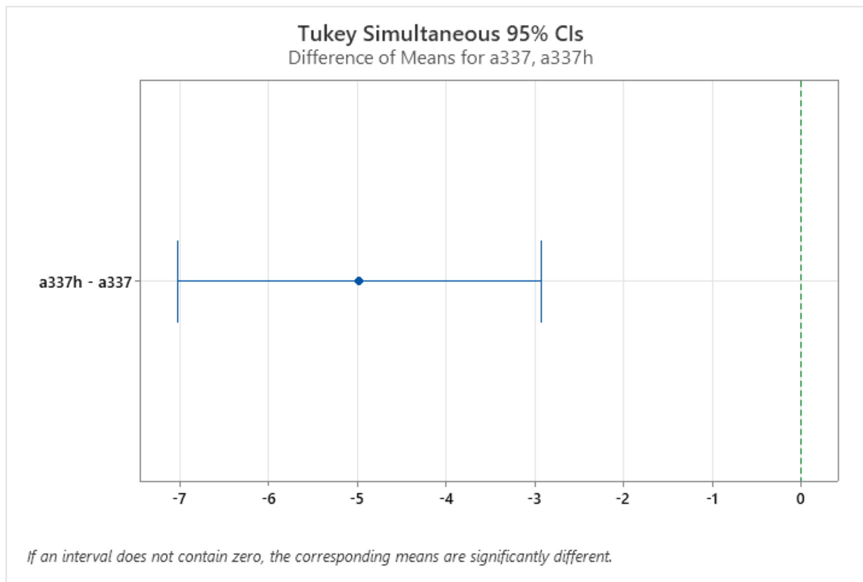
Factor	N	Mean	StDev	95% CI
a337	3	5.091	1.276	(3.644, 6.537)
a337h	3	0.11100	0.00484	(-1.33546, 1.55745)

Pooled StDev = 0.902355

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping	
a337	3	5.091	A	
a337h	3	0.11100		B

Means that do not share a letter are significantly different.



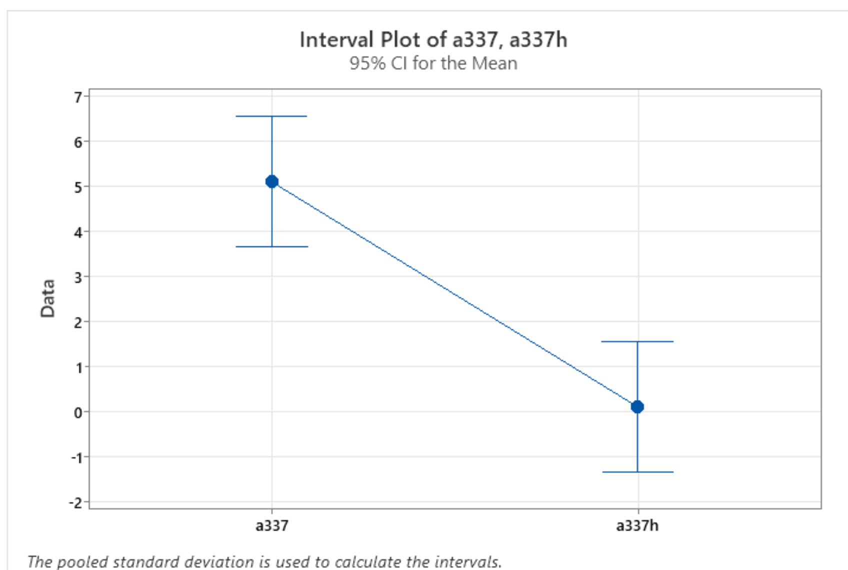


Figure C2: Example of One-way ANOVA with Turkey method using Minitab. For each sample, a One-way ANOVA test was carried out to determine the significant difference between amount of hydrolysis with and without heat treatment.