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The efficiency of novel enzymes in the removal of thermophilic bacilli biofilms from a stainless steel surface

A thesis presented in partial fulfilment of the requirements for the degree of
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Abstract

Background: Biofilms, an agglomerated microbial association produced from adherence of bacterial cells on a surface, pose substantial bacterial contaminations in the dairy industry. In particular, spore-forming thermophilic bacilli, which survive heat treatments, have been identified to be the abundant biofilm formers and contaminants in dairy products. The conventional cleaning regime, Clean-In-Place (CIP), widely adopted in the industry has been proven to lack hygienic performance on the removal of these biofilms. As a sustainable alternative, more researchers have been investigating enzymes such as proteases and amylase to use as a cleaner in the recent years. However, the majority of the studies conducted have cantered around the use of amylase and proteases at a basic pH at around 60°C on non-spore forming mesophilic or psychrophilic biofilms.

Aim: To examine the efficacy of novel enzymes (protease, amylase and endoglucanase) at an acidic pH and high temperature (85°C) in the removal of thermophilic bacilli biofilms (*A. flavithermus, B. licheniformis* and *G. stearothermophilus)* from a stainless steel surface.

Methods and results: *A. flavithermus, B. licheniformis* and *G. stearothermophilus* were initially screened for strong biofilm forming strains. Using Plackett-Burman experimental design, enzymes were screened on the strains on stainless steel coupons determined by plate and spore counts, and impedance microbiology. Following statistical analysis, amylase and protease for *A. flavithermus*, and protease and endoglucanase for *G. stearothermophilus* were found to be significant in reducing bacterial cells. As no statistical analysis could be performed on *B. licheniformis* with the data obtained, protease and amylase were selected to be tested on *B. licheniformis* strains for further microscopy analysis. The enzymes were further examined on biofilms developed in a biofilm reactor in a batch mode based on plate and spore counts, impedance microbiology and microscopic techniques (epifluorescence microscopy and Scanning Electron Microscopy). The results demonstrated the efficiencies of amylase, protease, and the combination of amylase and protease for *A. flavithermus* and *B. licheniformis*, and endoglucanase for *G. stearothermophilus*. As a conformational experiment, the enzymes were investigated on the removal of biofilms formed in the continuous flow biofilm reactors, and the results confirmed and attested to the efficiencies of the enzymes.

Conclusions: The results revealed that amylase, protease (CB14057) and the combination of amylase and protease for *A. flavithermus and B. licheniformis*, and endoglucanase (CB13961) for *G. stearothermophilus* had a significant impact on reducing biofilm cells (2-3 log reduction depending on the flow system adopted) and Extracellular Polysaccharides Substances (EPS).

Significance and impact of the study: The present study widens the knowledge in the role of enzymes on the removal of spore-forming thermophilic bacilli biofilms in dairy processing. The structures and biofilm matrices of the tested thermophiles are yet to be known, however, this will form the basis of the future research directions.

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Chapter 1. Introduction

Biofilms are an agglomeration of microbial cells characterized by attachment to a surface and production of a matrix consisting of bacterial cells, Extracellular Polysaccharide Substances (EPSs), proteins, and DNA (Austin & Bergeron, 2009; Marchand et al., 2012). Once developed, biofilms are highly resistant to antimicrobial activities and cleaning or disinfection procedures, posing a significant safety concern in the food industry (Austin & Bergeron, 2009). In particular, endospore-forming thermophilic bacilli such as *A. flavithermus, B. licheniformis* and *G. stearothermophilus* are abundant microorganisms present in dairy products, acting as a hygienic indicator due to their thermo-stability, and the ability to form spores and grow at a wide temperature range (Burgess et al., 2010). Despite the substantial implications of spore-forming dairy thermophilic bacilli biofilms, the understanding of the structure of the biofilms still remain at a great uncertainty.

As a means to regulate contaminations associated with biofilms and prevent potential bacterial adherence, cleaning regimes such as Clean-In-Place (CIP) has been widely used in the dairy industry (Maukonen et al., 2003). However, the standard CIP practice has a detrimental impact on the environment and many previous studies have demonstrated unsatisfactory hygienic performance of the procedure on the cleaning efficacy (Bremer, Fillery, & McQuillan (2006); Hinton, Trinh, Brooks, & Manderson (2002). As a consequence, alternative management schemes including the use of enzymes have gained much attention over the past years. Several studies have investigated proteases and amylases, each hydrolysing and degrading protein and polysaccharides, as enzyme cleaners on biofilms and subsequently attested to their efficiency in removing the biofilm cells. The novel enzymes used in the study from CinderBio that act at a low pH and higher temperature at 85°C may pose different implications from the conventional enzyme applications at a basic pH and lower temperature. Previous research conducted by Yang (2019) investigated the efficacy of proteases, amylase and endoglucanase (CinderBio) on biofilms of *Listeria monocytogenes* and *Cronobacter sakazakii* formed on dairy manufacturing surfaces (stainless steel). The results demonstrated the ability of proteases and endoglucanase in decreasing the biofilms cells developed in a plastic microtiter plate and on stainless steel coupons of *L. monocytogenes* and *C. sakazakii* under static and shear conditions.

Prior to the study by Yang (2019), a vast majority of the research conducted on this area focussed on enzyme cleaning applications at a basic pH and temperature range of 60°C on non spore-forming mesophiles or psychrophiles. Further, endoglucanase, a cellulose hydrolysing enzyme (linear polysaccharide), has not yet been sufficiently studied on spore-forming thermophiles in dairy, or in wider food applications. Their ability to remove attached spore-forming thermophilic bacilli biofilm cells in dairy systems is questionable and their synergistic effect when combined is yet to be known.

Therefore, the purpose of the present research is to examine the efficacy of novel enzymes (proteases, endoglucanases and amylases) on the removal of spore-forming thermophilic bacilli biofilms (*A. flavithermus, B. licheniformis* and *G. stearothermophilus*) formed on dairy manufacturing stainless steel surfaces.

The objectives of the study are -

i) To screen strains based on their biofilm forming capabilities for further enzyme cleaning examinations

ii) To preliminarily screen enzymes using Plackett-Burman design on biofilm removal of the thermophiles formed on SS coupons in a microtiter plate

iii) To examine the screened enzymes on the removal of biofilms developed in CDC biofilm reactors under a batch system

iv) To carry out conformational tests of the screened enzymes on biofilms formed in CDC biofilm reactors under a continuous flow system.

It is hypothesized that any of the enzymes tested (proteases, endoglucanases and amylases) will be able to achieve a satisfactory level of biofilm removal of *A. flavithermus, B. licheniformis* and *G. stearothermophilus*) formed on stainless steel coupons in dairy processing.

Chapter 2. Literature Review

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2.1. Introduction

Biofilms, a significant source of contaminations in dairy processing, have been gaining much research interest in the recent years due to product quality challenges, and the economic viability and sustainability of the industry (Flint et al., 2020). A large and growing body of literature has well established that common bacterial contaminations the dairy industry confronts are the genus *Enterobacter, Listeria, Lactobacillus, Micrococcus, Streptococcus, Bacillus* and *Pseudomonas* (Marchand et al., 2012; Srey, Jahid, & Ha, 2013). In particular, spore-forming and non-pathogenic thermophilic bacilli such as *Bacillus licheniformis* (Crielly, Logan, & Anderton, 1994), *Geobacillus stearothermophilus*, and *Anoxybacillus flavithermus* (Burgess, Brooks, Rakonjac, Walker, & Flint, 2009; Burgess, Flint, & Lindsay, 2014) were identified to be the abundant bacteria present in dairy products including raw and pasteurized milk or milk powder. *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* grow rapidly over a wide temperature range (40°C to 65°C) and facilitate biofilm formation and sporulation, resulting in spoilage and rancid flavor development through acid and enzyme productions (Burgess, Lindsay, & Flint, 2010). The conventional chemical-based cleaning regime widely adopted in the dairy industry, Clean-In-Place (CIP), has frequently been associated with inadequate hygienic performance in the removal of biofilms in New Zealand (Bremer et al., 2006; Burgess et al., 2009; Flint et al., 2020). Consequently, enzymes have been emerging as a valuable alternative cleaner by degrading EPS and biofilms (Lequette, Boels, Clarisse, & Faille, 2010). However, to date, there still remains much uncertainty and ambiguity in understanding spores and components in the biofilm matrices, particularly thermophilic biofilms, and the use of heat-tolerant enzymes in the removal of the biofilms in dairy processing.

The present literature review will address published articles and studies of the characteristics of thermophilic bacteria in the dairy industry, biofilms and spores of thermophiles, factors affecting the biofilm formation, control of biofilms, and enumeration methods to quantify the biofilm cells. It is critical to understand the mechanism of biofilm formations of dairy thermophilic bacilli, and the control of biofilms to mitigate the contamination. Thus, the review will form the basis of the enzymatic treatment approach to the removal of thermophilic bacilli biofilms developed in various growth environments.

2.2. Biofilms

Biofilm is a clustered microbial community generated from adherence of cells on a surface and production of Extracellular Polysaccharide Substances (EPSs), proteins, DNA and bacterial cells (Austin & Bergeron, 2009; Marchand et al., 2012). Biofilms form rapidly on dairy processing stainless steel surfaces and are highly resistant to antimicrobial substances (Austin & Bergeron, 2009), cleaning and disinfection (Parkar, Flint, & Brooks, 2003), resulting in inevitable microbial contamination in the final product (Srey et al., 2013). Biofilms developed by thermophilic bacilli are referred to as process biofilms, generally comprised of one species in less dense matrices, owing to selective pressures of the processing parameters (Burgess et al., 2009). Previous research has demonstrated that *B. licheniformis, G. stearothermophilus*, and *A. flavithermus* are the three prevalent species to cause biofilm formations on a SS surface in a skim milk powder manufacturing plant (Sadiq et al., 2017).

2.2.1. Formation of biofilms in dairy systems

Marchand et al. (2012) described the biofilm formation mechanisms in the dairy manufacturing context, which involves multiple stages including conditioning layer, bacterial adhesion, bacterial growth, and biofilm expansion (Figure 1). Biofilm development in a dairy system is initiated by an accumulation of organic matter (i.e., milk components) on a metal surface, which subsequently modifies the surface properties such as hydrophobicity and electrostatic charges. The altered surface characteristics may later influence bacterial colonization where single vegetative cells are attracted to the surfaces to develop reversible bonds (Marchand et al., 2012). The irreversible attachment is promoted by the transition of the weak interactions of the bacteria to a permanent bonding resulting from the EPS development (Srey et al., 2013). The established EPS then proliferates through embracing surrounding planktonic cells for structural maturation and organization, eventually dispersing the planktonic cells upon external and internal disturbance including shear, enzymatic degradation or release of surface-binding protein (Srey et al., 2013). While early studies speculated that the minimum required incubation time for detectable biofilm growth was 48 hours (Flint, Bremer, & Brooks, 1997; Hood & Zottola, 1995), the biofilm formation of thermophilic bacilli has been found to develop rapidly. Flint et al. (2001) and Burgess et al. (2009) confirmed the biofilm formation and sporulation of the most prominent thermophilic bacilli in dairy products, *G. stearothermophilus* and *A. flavithermus* respectively, within 6 – 8 hours at 55°C in a laboratory continuous flow reactor.

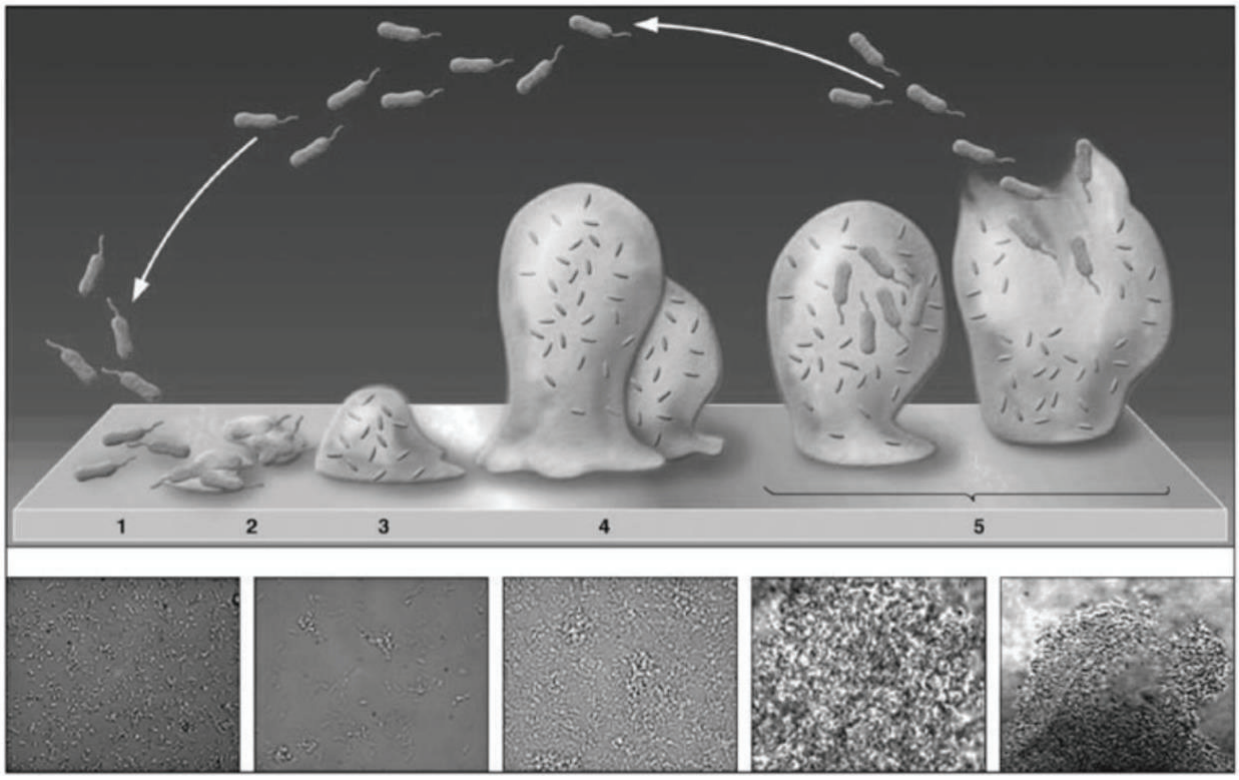


Figure 1. Stages of bacterial biofilm development. Adapted from Marchand et al. (2012). Stage 1: Reversible initial attachment of cells to the surface. Stage 2: Production of EPS resulting in more firmly adhered "irreversible" attachment. Stage 3: Early development of biofilm architecture. Stage 4: Maturation of biofilm architecture. Stage 5: Dispersion of single cells from the biofilm. The bottom panels show each of the 5 stages of development represented by a photomicrograph of *P. aeruginosa* when grown under continuous-flow conditions on a glass substratum

2.2.2. Spores in thermophilic bacilli biofilms

While limited knowledge still exists on spores and thermophilic bacilli biofilms in a dairy manufacturing system, Parkar, Flint, Palmer, & Brooks (2001) considered the biofilm formation of spore forming thermophilic bacilli to be a multifactorial process. Thermophilic bacilli spores and vegetable cells have been proven to attach to SS, the most common manufacturing equipment surface in the dairy industry. Spores adhere to SS more rapidly than vegetative cells due to their relatively higher hydrophobicity (Flint, Palmer, Bloemen, Brooks, & Crawford, 2001; Parkar, Flint, Palmer, & Brooks, 2001), demonstrated by certain spores of the *Bacillus* species (i.e., *G. stearothermophilus*) and strains with low hydrophobicity being less attracted to SS surfaces (Flint et al, 2001). Moreover, according to Burgess et al. (2009), it is believed that once thermophilic bacilli vegetative cells and spores are attached to the surface, the spores germinate, and the vegetative cells reproduce to form a biofilm complex. The authors subsequently postulated that sporulation may commence within the established biofilm, eventually leading to shedding of predominantly vegetative cells which are likely to contaminate the process liquid. These released spores and vegetative cells may also attach to other surfaces to produce and extend the biofilms as depicted in Figure 1 (Stage 5) (Flint et al, 2001).

In contrast to the previous research conducted on mesophilic bacilli (Burgess et al., 2014), Burgess et al. (2009) suggested that the biofilm formation of thermophilic bacilli and sporulation, shown by *A. flavithermus*, occur simultaneously at a fast rate. The researchers also indicated that the spores account for 10 – 50% of the *A. flavithermus* biofilm matrix formed in 8 hours. Thus, the spore-forming thermophilic bacilli biofilms encapsulating more adhesive, and heat and chemical-tolerant endospores exhibit great resistance to cleaning regimes, posing significant danger to the dairy industry.

2.2.3. Factors influencing biofilm attachment and thermophilic contamination in dairy manufacturing plant

Biofilm development in the dairy industry is influenced by a number of factors such as the specific bacteria strains, properties of a growing surface, surface charge, hydrophobicity, flow, nutrient composition, pH, and temperature (Srey et al., 2013).

2.2.3.1. Biofilm growing material

The formation of biofilms differs significantly and is contingent upon properties of a growing surface. A considerable amount of literature reports the use of microtiter plates for the development of biofilms on a laboratory scale (Karaca, Buzrul, & Coleri Cihan, 2019; Ozel et al., 2017; Stepanovic, Cirkovic, Ranin, & Svabic-Vlahovic, 2004), owing to its ability to produce high throughput screening of multiple strains in a short time (Djordjevic, Wiedmann, & McLandsborough, 2002). Moreover, Djordjevic et al. (2002) demonstrated that higher biofilm adhesion was observed on PVC microtiter plate than SS. This is in agreement with previously published studies, and many researchers ascribed the finding to the discrepancies in the hydrophobicity, where plastics have a higher hydrophobicity than SS influencing the number of biofilm bacteria adhering (Adetunji & Isola, 2011; Srey et al., 2013).

The dairy industry uses stainless steel for almost all manufacturing equipment. However, a large volume of published studies have discovered the growth of thermophilic bacilli biofilms such as *G. stearothermophilus* (Flint et al., 2001), *A. flavithermus*, and *B. licheniformis* on SS during dairy product manufacture (Burgess et al., 2014). Parkar et al. (2003) examined the biofilm attachment of *A. flavithermus* to SS using epifluorescence microscopy and impedance microbiology, and confirmed that the new, cleaned, and sterilized SS coupons were prone to biofilm formation in pasteurized skim milk within 6 hours. The authors accordingly speculated that while robust cleaning regimes for food-contact surfaces are also crucial, ideally preventing biofilm formation is a better strategy.

2.2.3.2. Flow conditions

The structure and detachment of biofilms are influenced by the flow rate of the liquid passing over the biofilm (Parkar et al., 2003). For example, biofilms grow denser under high rather than low shear conditions (Stoodley, Sauer, Davies, & Costerton, 2002).

The CDC (Center of Disease Control) biofilm reactor allows biofilm formation under a shear in both batch operation, and in a continuous flow system, which is more adaptable to the industry by emulating and creating a similar manufacturing environment in which the biofilms are formed. The apparatus enables characterization of biofilm structure and morphology, and assessment of the impact of antimicrobial agents (Donlan & Costerton, 2002). Goeres et al. (2005) attempted to statistically evaluate the CDC biofilm reactor for growing biofilms of *Pseudomonas aeruginosa* and concluded that the CDC biofilm reactor is a reliable tool for developing a range of bacterial biofilms in the laboratory. The authors also criticized the statement by in Stoodley et al. (2002) that biofilms developed dense elongated cell clusters under a high shear, suggesting that each tested biofilm did not originate from the same growth environment and thus a legitimate comparison could not be established. Nonetheless, shear is thought to have an impact on the adhesion of biofilms to some extent as proposed by Parkar et al. (2003), who confirmed that the attachment is enhanced in both laminar and turbulent flow conditions in comparison to static conditions (Parkar et al., 2003), possibly owing to the increased adhesion of the bacterial cells (Donlan & Costerton, 2002).

2.2.3.3. Availability of nutrients

While operating the CDC biofilm reactor under a continuous flow closely resembles the biofilm formation in the industry application, it is resource intensive due to the need for a continuous medium supply and labour intensive as different flow rates are required for every strain examined, based on the doubling time of each bacterium. Hence, biofilm development in the CDC biofilm reactor using shear but in a batch mode may produce useful results with limited resources and time. Using the CDC biofilm reactor in the batch mode, the medium undergoes changes in the chemical composition until all the limited nutrients are consumed, whereas in the flow system, there is a constant replacement of media, and biomass allows the system to achieve a steady state. (Murga, Miller, & Donlan, 2001). Numerous studies have reported that nutrient content in the medium is one of the aspects influencing biofilm attachment and growth (Austin & Bergeron, 2009; Flint et al., 1997; Parkar et al., 2003). Similarly, Ronner & Wong (1993) showed a longer lag time for bacterial growth in a one-fifth diluted nutrient broth.

Murga et al. (2001) and Yoon & Lee (2017) used the CDC biofilm reactor in the batch mode, however, the purpose was to promote the initial colonization of the bacteria on the surface or culture growth prior to the defined analysis. To date, there is no or a substantial lack of studies on the investigation of biofilm formation in a CDC biofilm reactor in a batch mode or its correlation to its counterpart continuous system.

2.3. Thermophilic bacteria

2.3.1. Characteristics of thermophilic bacteria

Thermophilic bacilli such as *Geobacillus stearothermophilus, Anoxybacillus flavithermus*, and *Bacillus* species (*Bacillus licheniformis*) are thermo-stable microorganisms prevalent in the dairy industry, as well as paper mills, canning, juice pasteurization, and gelatin production industries (Burgess et al., 2010). Thermophilic bacilli are non-pathogenic contaminants of the dairy industry (Burgess et al., 2010). They indicate problems in manufacturing plant hygiene by growing rapidly over a wide temperature growth range (40°C to 65°C) and producing endospores which may compromise quality of the dairy products (Burgess et al., 2009; Burgess et al., 2010). These spore-forming microorganisms may also facilitate spoilage in foods as a result of acid and enzyme production following spore germination (Burgess et al., 2009; Burgess et al., 2014). It is believed that thermophile contaminations in a finished dairy product originates from a small number of thermophiles present in raw milk, which then propagate and proliferate as biofilms in dairy manufacturing plant releasing bacteria and spores, leading to high numbers of thermophiles (10^4 – 10^6 cfu/g) in the final product (Burgess et al., 2010; Sadiq et al., 2017). The dramatic reduction in the microbial quality of the milk products following a short residence time in the manufacturing plant indicates concerns associated with biofilms development during processing (Burgess et al., 2010; Flint et al., 2001; Srey et al., 2013).

G. stearothermophilus, A. flavithermus, and *B. licheniformis* undergo sporulation and form endospores that enable to bacteria to survive stress or extreme conditions. Table 1 summarises the characteristics of the thermophilic bacilli species of interest in this study. Thermophilic bacilli endospores are difficult to eliminate during dairy manufacturing processes due to their resistance to heat, shear, and chemicals (Marchand et al., 2012). While it has been known that sporulation of mesophilic bacilli is initiated by starvation, high cell density, or DNA damage (Cortezzo & Setlow, 2005), spore formation of thermophiles in dairy conditions still remain uncertain. Nonetheless, Palop, Maras, & Condon (1998) stipulated that certain minerals, calcium in particular, has been correlated with spore formation of thermophiles, as well as subsequent spore maturation by increasing the expression of genes (Oomes et al., 2009). Consequently, the presence of mineral salts and calcium in milk is a plausible possibility of triggering spore formation of these thermophiles in dairy systems.

Table 1. Summary of the characteristics of the thermophilic bacilli species investigated in the study

	Geobacillus stearothermophilus	***Anoxybacillus flavithermus***	***Bacillus licheniformis***
Obligate / facultative	Obligate thermophile	obligate thermophile	facultative thermophile
Respiration method	Aerobic or facultatively anaerobic	Facultatively anaerobic	Facultatively anaerobic
Spore forming	Yes	Yes	Yes
Growth temperature	40°C to 70°C	65°C to 72°C	50°C to 55°
Products	Milk powder	Milk powder	Milk powder
Location in plant	Evaporators and plate heat exchangers	Heat exchangers evaporators	UHT pilot plant

(Burgess et al., 2010) (S. Flint et al., 2001) (Sadiq et al., 2017) (Zeigler, 2014) (Burgess et al., 2009) (Burgess et al., 2009; Burgess et al., 2014) (Burgess et al., 2010). (Burgess et al., 2010; Crielly et al., 1994)

2.4. Control of biofilms in the dairy industry

Multiple biofilm control strategies such as the control of microbes before biofilm development, removal of biofilms using disinfectants, and inhibition of attachment of cells by selecting a surface which opposes and hinders bacterial attachment as proposed by Meyer (2013), could be adopted to prevent potential biofilm contamination.

Clean-In-Practice (CIP) describes a process in which a system is cleaned without dismantling equipment (Srey et al., 2013). Conventional CIP widely used in the food industry decreases surface tension, emulsify fats and dissolve denatured protein (Maukonen et al., 2003), and react with the EPS complex (Srey et al., 2013), thereby cleaning the surface and mechanically removing biofilms. The most common and destructive caustic cleaner in the dairy industry is sodium hydroxide (NaOH), which primarily removes proteins and carbohydrates (Blackman & Frank, 1996). However, an unsatisfactory hygienic performance of the standard CIP in eliminating the biofilm was consistently corroborated and substantiated by many researchers (Hinton, Trinh, Brooks, & Manderson, 2002; Lequette et al., 2010). Lelièvre, Antonini, Faille, & Bénézech, (2002) also observed 20-30% of *B. cereus* spore residues on the surface after 30 minutes treatment with NaOH.

The conventional chemical-based cleaning methods present a number of limitations such as a substantial environmental impact, the possibility of bacteria developing survival strategies to the chemicals through gradual mutation, and inadequate cleaning outcomes through the difficulties in penetrating the EPS of the biofilm. In the wake of this, alternative cleaning regimes, including enzymatic treatments, have attracted much attention in the recent years. Table 2 summaries advantages and disadvantages associated with the traditional CIP, as well as abundant enzymes commercially used in many fields (Lequette et al., 2010; Meyer, 2003).

Lequette et al. (2010) demonstrated that two serine proteases were more effective in removing cells and EPS of *Bacillus* biofilms than the alpha- amylase, which enhanced removal of *P.fluorescens* biofilms. The authors ascribed the discrepancies in the results to the significant variations in the heterogeneities of biofilms developed from each strain, and subsequently proposed that the use of a combination of enzymes on a range of bacterial species would be beneficial for the detachment of biofilms (Meireles, Borges, Giaouris, & Simões, 2016). Similarly, Eide, Homleid & Mattsson (2003) carried out a Life Cycle Assessment (LCA) on four CIP processes (alkaline/acid with hot water disinfection, one phase alkaline with acid chemical disinfection, enzyme with acid chemical disinfection and dissection by cold nitric acid) in a dairy system and found that the enzyme cleaning in small volumes at low temperatures was effective. However, the author did not address the nature of the enzyme (i.e., proteolytic or polysaccharide degrading) being tested. Further, most of the publications predominantly focus on the use of the enzymes at a basic pH and temperature around 60°C on mesophiles or psychrophiles. The use of endoglucanase, a cellulose (linear polysaccharide) hydrolyzing enzyme, as a cleaner has not been studied or extremely little is known about the removal of thermophilic bacilli biofilms. Parkar et al. (2003), as well as other researchers such as Sadiq et al. (2017) stated that some thermophilic bacilli are abundant producers of heat-stable proteinases which are distributed in the EPS, destroying cell surface proteins responsible for the initial attachment of biofilm cells. Consequently, the efficiencies of the enzymes at a low pH and high temperature (85°C) (likely to be found in thermophilic bacilli biofilms) in the removal of biofilms of spore-forming thermophiles are questionable.

Table 2. Benefits and limitations of CIP and enzyme treatments to remove biofilms in the dairy industry

Control strategies	Advantages	Disadvantages
CIP processes (e.g., NaOH)	• Highly efficient if specifically optimized as per the fouled deposits being treated • Multi-functional systems are efficient and economic • Efficiency of regimes can be increased by altering conditions such as temperature and concentration of detergents	• High water consumption • High energy usage to operate equipment • Single-use CIP units are expensive to operate • Process is time-consuming and any cleaning time is classified as downtime
Enzymes	• Environmentally friendly • Bacteriocins deriving from lactic acid bacteria have GRAS status (generally recognized as safe) • Wide antibacterial spectrum • Bacteriocins are not active and non-toxic to eukaryotic cells • Bacteriocins do not affect the gut microbiota	• Enzymes are highly specific and setting up a cocktail of enzyme against biofilms is time-consuming and expensive • Bacteria in biofilms have developed resistance to biocides • Bacteriocins do not always target the desired bacterial group • A complex food microbiota can reduce the efficacy of bacteriocins

Note. Reprinted from "The Prevalence and Control of Bacillus and Related Spore-Forming Bacteria in the Dairy Industry," by N. Gopal, C. Hill, P. Ross, T. Beresford, M. Fenelon, and P. Cotter, 2015, Front Microbiology, 6. Copyright 2015 by "Gopal, Hill, Ross, Beresford, Fenelon and Cotter.

2.5. Quantification and enumeration of biofilm cells

2.5.1. Total plate count

Various methods have been employed by researchers to quantify and enumerate adhered biofilm cells. The removal of biofilm cells using a mechanical shear including vortex or sonication and subsequent traditional plating methods, and microscopic techniques involve detachment and direct counting of cells (Djordjevic et al., 2002; Hood & Zottola, 1995). Jeong & Frank (1994) established that 97% biofilm cells were recovered by scraping with a Teflon spatula and the plate count method from a SS surface. While the standard plate count method is versatile and widely adopted, the approach presents several limitations including i) inconsistency in the results due to the incomplete detachment of the cells, ii) underestimation of the extent of contamination resulting from inadequately dispersed cells, iii) difficulty in detecting viable but non-culturable bacteria, and iv) a time-consuming procedure (Flint et al., 1997; Hood & Zottola, 1995). This in turn raises the need for introducing a rapid measure for detecting biofilm contamination adaptable to the industry.

2.5.2. Impedance microbiology

Impedance microbiology measures the impedance in the medium or on the surface of a pair of electrodes immersed in a growth medium and records the point the change reaches a set threshold (Gomez-Sjoberg, Morisette, & Bashir, 2005). Impedance microbiology is a rapid, easily manageable and highly sensitive system for monitoring bacteria and biofilm cells (Flint & Brooks, 2001; Gomez-Sjoberg et al., 2005; Silley & Forsythe, 1996). Impedance microbiology also circumvents the inaccuracy of physical removal of the adhered cells (i.e., vortex) (Silley & Forsythe, 1996). Despite the benefits related to impedance microbiology, the calibration measurements are primarily based on planktonic cells, owing to the lack of current knowledge in the biofilm growth curve, which may raise the need for a careful approach in interpreting the results when comparing against biofilm cells. Nonetheless, Flint & Brooks (2001) reported that the BacTrac 4000 was effectively able to detect *G. stearothermophilus* within 8 hours and concluded that the method was a promising alternative to plate counting for enumerating thermophilic bacilli species in food. Similarly, Mosteller & Bishop (1993) demonstrated that between the standard plate count, impedance microbiology and epifluorescence microscopy, impedance microbiology was identified to be the best enumeration method for *P. fluorescens, Y enterocolitica* and *L. monocytogenes* biofilms, as it took both reversibly and irreversibly attached bacteria into account.

2.5.3. Epifluorescence microscopy

Epifluorescence microscopy utilizes light emittance at a specific wavelength exerted by micro-organisms stained with fluorescence dyes (Donlan & Costerton, 2002). Acridine Orange is a prevalent fluorescent dye, producing green fluorescence when bound to DNA and red florescence when intercalated with RNA (Byvaltsev et al., 2019), thereby allowing differentiation of living and dead cells (Hood & Zottola, 1995). However, the authors later

reported variability and a lack of accuracy of Acridine Orange in the differentiation of live and dead cells. This finding is in accordance with (Blackman & Frank, 1996), who speculated that biofilm quantification using epifluorescence microscopy is correlated with an underestimation of biofilm cells from not accounting for the thickness, and also overestimation of cells due to the staining of some EPS. (Hood & Zottola, 1995) concluded that the ability of Acridine Orange to determine the state of cells is questionable and debatable. In spite of this, the authors postulated that epifluorescence microscopy using Acridine Orange may be effective to observe colonisation of a non-transparent surface.

2.5.4. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) has been used extensively to observe biofilm morphology. Hood & Zottola (1995) speculated that the microscopic techniques were more capable of enumerating low bacterial cells, whereas the standard plating method can enumerate higher contamination levels. In contrast, Austin & Bergeron (2009) examined biofilm development in dairies using SEM and ascertained that SEM failed to produce quantitative results for viable organisms, however, it provided an informative visual representation of the bacterial adhesion over the tested sample area. As Brew (1928) demonstrated that direct microscopic method led to higher counts, high numbers of viable bacterial cells need to exist for microscopic techniques to be capable of enumerating the cells, while standard plate count is relatively applicable to quantifying lower contamination levels. Microscopic assays are time-consuming in comparison to the other analysis methods such as using a microtiter plate, hence, it is worthwhile to establish prediction of biofilm development (i.e., screening) before carrying out an assessment by microscopic techniques (Djordjevic et al., 2002).

2.6. Conclusions

Thermophilic bacilli such as *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* pose substantial quality and hygiene concerns in the dairy industry as a consequence of the production of highly resistant and stable spores and biofilm formation on manufacturing plant surfaces (stainless steel). The multifactorial process of biofilm development is influenced by several factors such as surface properties, pH, medium, temperature, flow conditions and nutrient availability. Understanding the factors responsible for spore formation in a biofilm is not yet clear however, spores are able to attach rapidly on stainless steel and account for 10 - 50% of a thermophilic biofilm (Burgess et al., 2009), enabling the biofilms to exhibit great resistance to cleaning regimes.

Clean-In-Practice (CIP), a cleaning regime involving flushing the surface at high velocity without dismantling the system, has traditionally been adopted by the dairy industry, mainly utilizing NaOH under specific conditions. However, the standard caustic method has been shown to produce unsatisfactory results in some circumstances along with a substantial

environmental impact. Thus, enzymatic cleaning has gained attention as a plausible alternative. Proteolytic (proteases) and polysaccharide (amylase) can effectively remove the EPS of a number of bacterial species. Studies have predominantly centered around the use of enzymes at a basic pH at temperature around 60°C on mesophiles or psychrophiles. Endoglucanase (different type of the polysaccharide enzyme) has not yet been evaluated.

The standard plate count following removal of bacteria from a surface is a versatile and commonly used method for quantifying biofilm cells, however it is time-consuming and may lead to inconsistencies and an underestimation of cells. Impedance microbiology rapidly detects the change in impedance in the medium resulting from the growth of bacterial cells. The use of epifluorescence microscopy using acridine orange allows visualization of bacterial cells before and after cleaning and is an efficient method to observe biofilm on a non- transparent surface, yet it may be associated with variability and a lack of accuracy in the presence of EPS. It also lacks sensitivity where there are low numbers of cells present. SEM is also a widely used microscopy technique to acquire an informative visual representation of bacterial adhesion for qualitative examinations. The use of SEM allows demonstrations on where the biofilm is located on a surface, shape of the cells, indication of spores and amount of EPS present.

This study will use these methods to evaluate the use of novel enzymes that act at low pH and high temperature for the removal of thermophilic bacilli biofilms formed on stainless steel surfaces. Several enumeration and analysis methodologies will be employed for accurate and precise enumeration of biofilm cells, as well as to obtain a broader understanding of the biofilm composition and morphology.

Chapter 3. Materials and Methods

3.1. Experimental design

The experimental design for the present study is outlined in Figure 2 and detailed as follows:

A. Selection of strains involved screening of strains that were able to form biofilms in a 24-well microtiter plate on SS coupons. The best biofilm producing strains (two strains) for each *A. flavithermus, B. licheniformis*, *and G. stearothermophilus* were selected for further analysis.

B. i. Biofilms prepared from the selected strains were examined with various enzyme treatments and their combined effects to observe bacterial cell reduction. The experiment was conducted using a Plackett-Burman design, followed by a full factorial design in the subsequent experimental phase (B.ii.). Three microbial tests were used to enumerate and quantify the bacterial cells.

B. ii. The enzymes which significantly decreased biofilm cells on the coupons were thoroughly assessed in a biofilm reactor run in batch mode. The CDC biofilm reactor which provided constant shear, representative of an industrial system, was used to grow biofilms. For qualitative image analysis of the stainless steel surfaces, epifluorescence microscopy was used along with acridine orange fluorescence dye.

C. Conformational tests were carried out to substantiate the efficiency of the screened enzyme treatments in the CDC biofilm reactor under a continuous flow system. The continuous flow is the relevant operational system applicable to the industry for developing biofilms, which allowed passage of the medium continuously to ensure that there was no net growth in the milk that would affect the results. In addition to the epifluorescence microscopy, Scanning Electron Microscope (SEM) was also used to further demonstrate and visualize the biofilm morphology and composition of the biofilm.

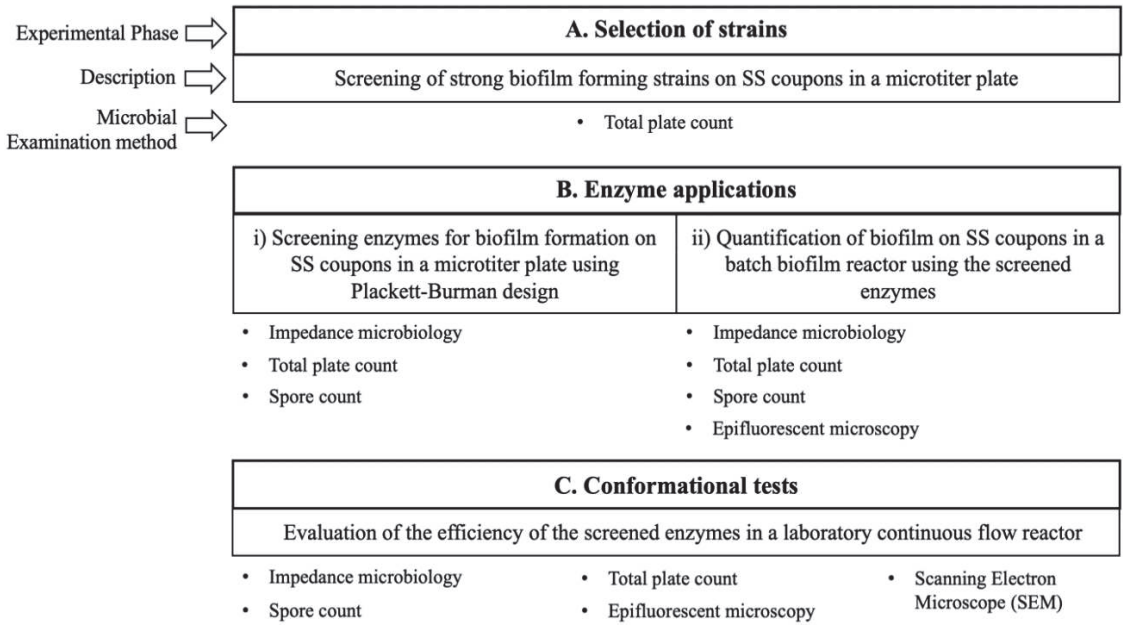


Figure 2. Experimental design used in the study

3.1.1. Selection of strains

Table 3 below summarizes the strains and the origins for *A .flavithermus, B. licheniformis, G. stearothermophilus* species used in the research.

Table 3. Bacteria strains and origins used in the study

Bacteria	Strains	Source
Anoxybacillus flavithermus	P2	Milk powder manufacturing plant
	T2	Milk powder manufacturing plant
	B12	Milk powder
	T18C	Milk powder manufacturing plant
Bacillus licheniformis	C55C11	Whey protein
	C55C01	Whey protein
	D55C11	Whey protein
Geobacillus stearothermophilus	P3	Evaporated milk
	A1	Evaporated milk
	D1	Evaporated milk

Cultures were maintained at -80°C in Cryobeads banks (Mast Group Ltd, Liverpoo, UK). For use, one bead was transferred and grown overnight on Milk Plate Count Agar (MPCA, OXOID, Hampshire, England) using 16 streak technique, from which pure colonies were taken and subcultured in 9 ml of Trypticase Soy Broth (TSB) (BBL, Becton Dickinson, Cockeysville, MD, USA). TSB has been known to be a highly versatile medium, capable of growing spore-forming thermophiles (Karaca et at, 2019). The broth was incubated for 8 hrs at 55°C, 40°C and 60°C for *A. flavithermus, B. licheniformis,* and *G. stearothermophilus* respectively, and vortexed to allow uniform distribution of cells. The incubation periods for the bacteria were determined based on a preliminary experiment to ascertain their growth behaviors (not presented in the study). After incubation, Optical Density (O.D) of the growth was measured at a wavelength 600nm using Spectrostar Nano (BMG Labtech, Ortenberg, Germany) and adjusted to approximately 0.5 O.D to achieve a cell density of 10^5 – 10^5 CFU/ml to be used as a starting culture.

For coupon preparation, SS coupons (304 grade with a 2B surface finish) with r = 12.7 mm, thickness = 3.8 mm and total A = 4.05 cm^2 (Biosurface Technologies Cooperation, USA) were washed in 1% NaOH (Merk, Damstadt, Germany) for 10 minutes, followed by rinsing with distilled water. The coupons were then immersed in acetone (Univar, Illinois, USA) for 10 mins to remove any grease, rinsed by distilled water, and autoclaved. The autoclaved SS coupons were then aseptically placed into each of the wells in a 24-well microtiter plate. 37.5ml (1.5%) of 8 hr cultures of *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* were inoculated into each well containing a SS coupon and 2.5ml of Anchor UHT whole milk (Fonterra, NZ) for 12 hr incubation at the appropriate temperature at 100 rpm. Following incubation, the coupons were removed from the microtiter plate and rinsed in 40 ml sterile distilled water three times to allow detachment of vegetative cells. All experiments were conducted in triplicate.

3.1.2. Enzyme applications

3.1.2.1. Screening enzymes for biofilm removal on SS coupons in a microtiter plate using a Plackett-Burman design

Biofilm development followed Section 3.1.1. After rinsing the biofilm coupons, the coupons were treated with enzyme solutions. For enzymatic solution preparation, one amylase, two proteases (CB14057 and CB23726) and two endoglucanase enzymes (CB13366 and CB13961) (CinderBio, USA) were diluted 100 times in phosphate citric acid buffer. For mixture enzymatic solutions, equal amount of each enzyme was added to the buffer to achieve 1:100 dilutions (i.e., for a solution containing two different enzymes, 0.5ml of each enzyme was added into 100ml of phosphate citric acid buffer). To make the phosphate citric acid buffer, 2.84g of Sodium phosphate dibasic (Na_3PO_4) (Univar, Illinois, USA) and 7.69g of citric acid (Univar, Illinois, USA) was dissolved in 1 litre of distilled water, after which the pH was adjusted to approximately 3.0 and autoclaved. The enzyme solutions were stored in a water bath at 85°C for future use. Table 4 below displays a Plackett-Burman design generated by Design of Experiments (DOE) on Minitab 18 (Pennsylvania, USA). Biofilm coupons were immersed and treated in 25ml vials containing 10ml of the enzyme solutions for 20 minutes at 85°C in water bath. In addition to the Plackett-Burman runs, examinations on biofilms coupons without any treatment (before cleaning), with 85°C sterile water (control), and with treatment with 1% NaOH were carried out in conjunction with the enzymatic cleaning to establish comparison. All examinations were conducted in duplicate

Table 4. Plackett Burman design generated

Number	Amylase	Protease (CB14057)	Protease (CB23726)	Endoglucanase (CB13366)	Endoglucanase (CB13961)
1	-1	1	1	-1	1
2	-1	1	-1	-1	-1
3	-1	-1	-1	1	1
4	1	1	-1	1	-1
5	-1	1	1	1	-1
6	1	-1	-1	-1	1
7	1	1	-1	1	1
8	-1	-1	-1	-1	-1
9	-1	-1	1	1	1
10	1	-1	1	-1	-1
11	1	1	1	-1	1
12	1	-1	1	1	-1

3.1.2.2. Quantification of biofilm on SS coupons in a batch biofilm reactor using the screened enzymes

For biofilm development under shear in a dynamic state, a CDC biofilm reactor (Biosurface Technologies Corporation, USA) was used (Figure 3). For the reactor to operate in a batch system and to prevent any bacterial contamination via air, the outlet of the reactor and air tubing on the top were thoroughly blocked using a 45µm filter and stopper. The reactor with SS coupons fitted into the cylindrical Teflon holder was autoclaved prior to use. Six mL (1.5%) of 8hr cultures of the three thermophiles grown in TSB were inoculated into the reactor containing 400ml of Anchor UHT milk. The reactor was then placed on a stir plate set to an appropriate temperature with a rotating speed of 100 rpm for 24 hour incubation. The incubation time increased from 12 hours in the microtiter plate to 24 hours in the reactor to allow a greater degree of bacterial adherence and hence more pronounced cleaning efficiency of the enzymes following treatment. After incubation, the Teflon holders were separated from the reactor and the SS coupons were removed and washed in 40ml of sterilized distilled water three times for microbial enumeration and analysis. All experiments were conducted in triplicate.

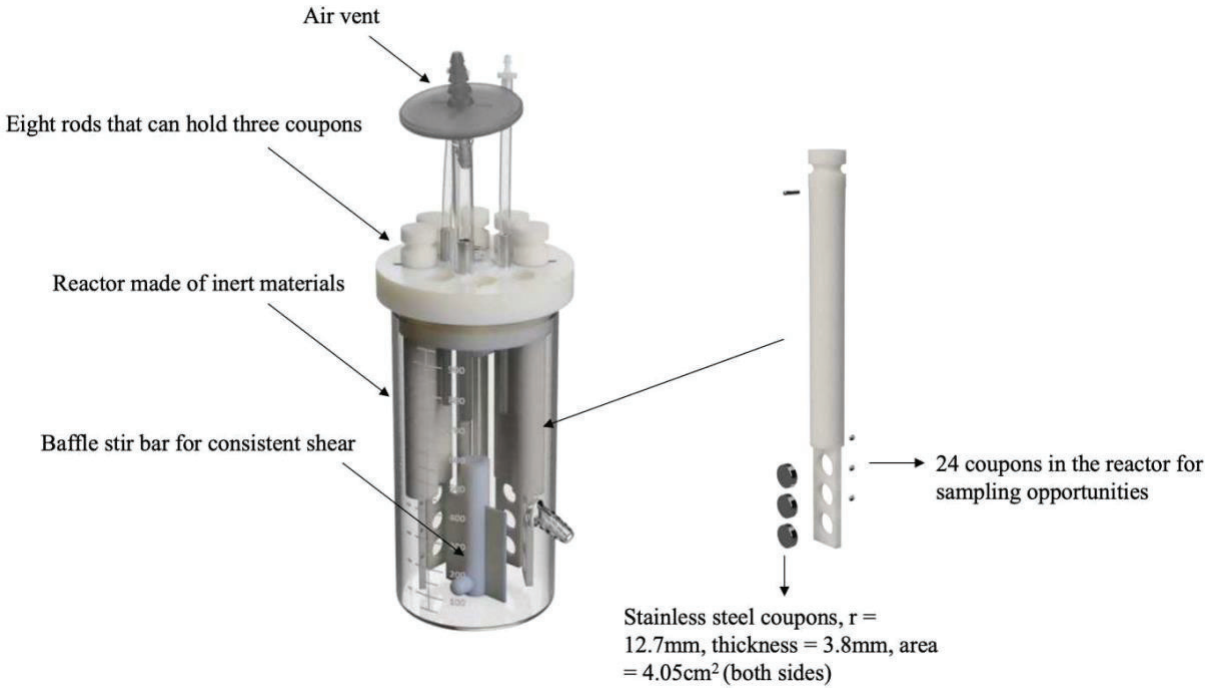


Figure 3. CDC biofilm reactor used to develop biofilms

3.1.3. Conformational tests

Figure 4 is a schematic diagram of the continuous flow CDC biofilm reactor. The UHT whole milk was prepared from whole milk powder (Fonterra, Palmerston North, NZ) at the FoodPilot Massey University. The milk powder was reconstituted in cold water using FP004 Cowles mixer (Massey University, Palmerston North, New Zealand) to produce 11% milk powder at a mixer speed 35 rpm and was hydrated for 20 minutes. The milk was then homogenized (Rannie, Copenhagen, Denmark) at 100pa and 50pa and processed through UHT equipment (Massey University, Palmerston North, New Zealand). The UHT milk was collected through a laminar cabinet into previously autoclaved 20L plastic cans.

300 ml of 8 hr cultures of the three thermophiles grown in TSB were inoculated into each can containing 20L of the UHT milk (≈10^3-10^4 CFU/ml). The UHT milk can was stored in the cold room and the milk was pumped (MasterFlex, Illinois, USA) at the calculated flow rates through a rubber tube (MasterFlex, Illinois, USA) to the reactor on a hot plate set to the appropriate temperature and agitation of 100 rpm.

For the flow rate calculations, doubling times of the three thermophiles were measured from growth curves and this was used to determine the flow rate. 8 hr cultures of *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* grown at 55°C, 40°C, and 60°C in TSB were each inoculated into three biofilm reactors without SS coupons fitted, which contained 400ml of UHT milk. The reactors were then placed on hot plates heated to the specified temperatures in a laminar flow cabinet. Starting from time zero, 1ml of samples were taken every 2 hours up to 12 hours from each of the three reactors aseptically, followed by 10-fold serial dilutions and droplet plating in triplicates on MPCA. The plates were incubated for 24 hours for plate count, and the results in Log 10 CFU/ml were plotted against time to obtain a growth curve. Based on the exponential phase determined from the growth curves, doubling time was calculated as shown in Equation 1.

$$\ln\left(\frac{C_f}{C_i}\right) = k(t_f - t_i) \qquad \text{Doubling time} = \frac{\ln 2}{k}$$

Equation 1. Rate constant and doubling time equations

Where k is rate constant, C_f is the final concentration in the exponential phase, C_i is the initial concentration in the exponential phase, t_f is the final time that reaches C_f, and t_i is the initial time. Flow rates were established such that the total volume of the milk in the reactor (approximately 430ml) was replaced with fresh milk in the time taken for the numbers of bacteria to double.

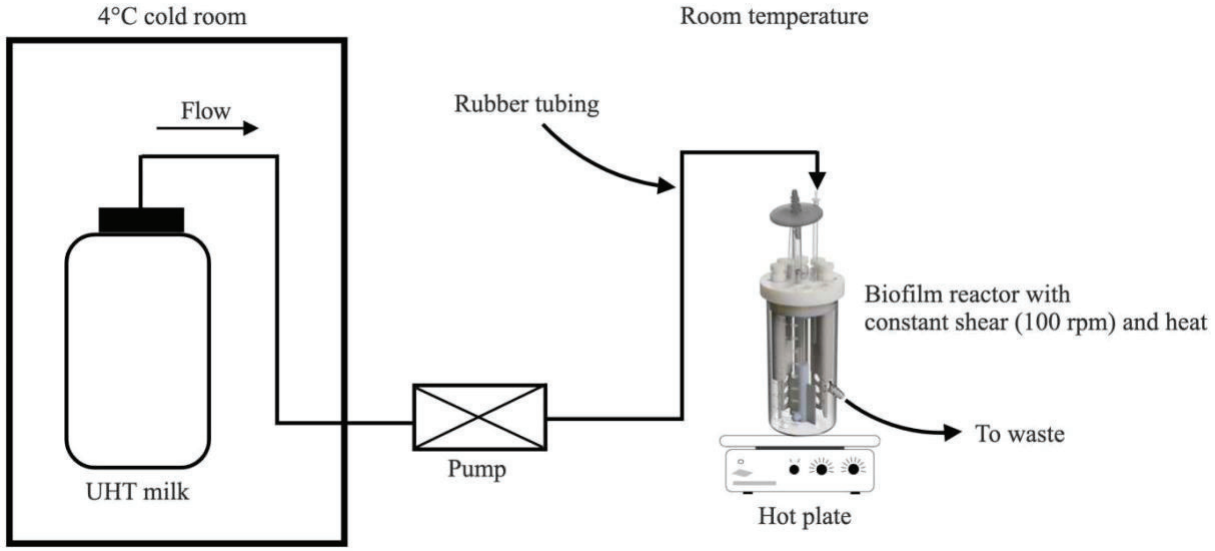


Figure 4. Schematic diagram of the continuous flow CDC biofilm reactor

3.2. Microbial examination

3.2.1. Plate count of biofilm cells attached and grown on the SS surfaces

SS coupons were rinsed in sterile distilled water and placed in 25ml vials containing 10ml of 0.1% Peptone Water (PW) (Merk, Damstadt, Germany) and 15g of 3.7 – 4.1mm glass beads (Fisher scientific, Leicestershire, UK). The vials containing the SS coupons were mixed by vortex for 1 min for biofilm cell detachment. After vortex mixing, 10-fold serial dilutions were carried out in 9ml of 0.1% PW for droplet plating (10 μl) in triplicate on MPCA. The plates were set to dry in a laminar flow cabinet and incubated at 55°C for 24 hrs.

3.2.2. Spore count

For examining spores present in the samples after plating the viable biofilm cells (Section 3.2.1.), the 25ml vials containing the SS coupons were placed in boiling water for 15 minutes to quantify spores that were able to survive the high temperature. This thermal treatment to deactivate spores was in line with Karaca et al. (2019), who treated thermophilic spores at 100°C for 15 mins. After heating, the vials were cooled down to room temperature prior to droplet plating in triplicate on MPCA. The plates were set to dry in a laminar flow cabinet and incubated at 55°C for 24 hrs.

3.2.3. Impedance microbiology

The BacTrac 4300TM (SyLab, Purkersdorf-Vienna, Austria) employs two detecting signals. M-value is a measure of the changes in impedance in the medium derived from microbial metabolism and resistance as a result of bacterial growth. The E-value identifies the changes in the impedance of the electrochemical double layers on the electrode surfaces. The threshold limits were set to 3% and 10% for M-value and E-value respectively, and the time taken to reach the defined threshold values were recorded and used as a measure of the extent of contamination and presence of biofilm cells.

3.2.3.1. Calibration

Figure 5 depicts the schematic diagram of the BacTrac calibration for measuring biofilm cells. 8 hr cultures of *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* grown at 55°C, 40°C, and 60°C in TSB were serially diluted in 9 ml of 0.1% PW. Each dilution was mixed by vortex and plated on MPCA using droplet plating in triplicate, and also incubated in BacTrac vials containing 10ml of TSB in triplicate. The plates were incubated for 24 hours at 55°C and the BacTrac vials were incubated at the appropriate temperature (the identical growing temperatures in TSB) for 24 hours for the change in impedance measurement. As each detection signal is applicable to a particular bacterium, the detection system (E-value or M- value) that exhibited reproducible growth curves was selected for the study for each strain. The plate count in Log10 CFU/ml was graphed against the time taken for impedance change to reach the set threshold values, from which the calibration equation was obtained. The calibration was later used to convert the BacTrac threshold times into the equivalent Log10 CFU/ml for quantifying biofilm cells.

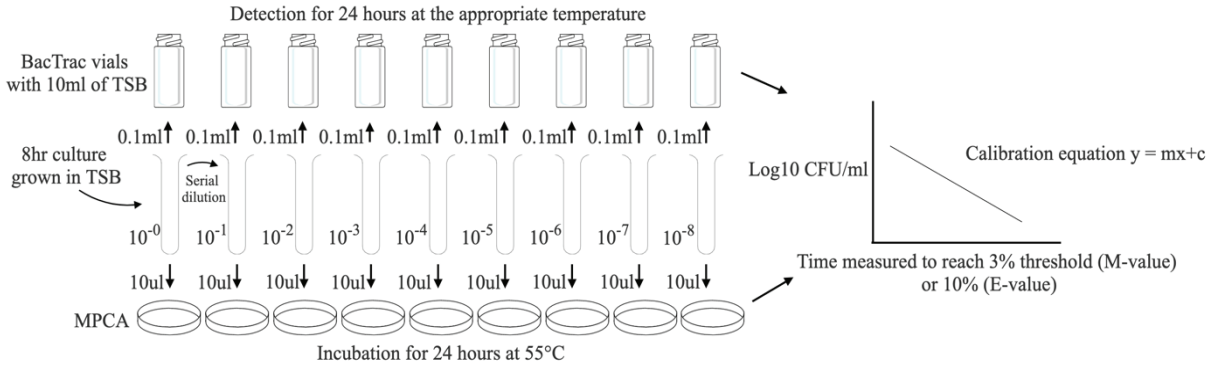


Figure 5. Schematic diagram of developing a calibration curve using BacTrac 4300

3.2.3.2.Measurement of biofilm cells on coupons

Rinsed SS coupons were each placed in BacTrac vials containing 10ml TSB and incubated for 24 hours at the appropriate temperatures for the detection of impedance change to reach 3% threshold for M-value and 10% threshold for E-value (depending on the curves produced, one detection system was selected). The experiments were conducted in duplicate for screening the enzyme treatments in a microtiter plate (Section 3.1.2.1.) , and in triplicate for examining the biofilm cells in a biofilm reactor (Section 3.1.2.2.) , and for the confirmational run using the continuous flow system (3.1.3.).

3.2.4. Epifluorescence microscopy

10g of the fluorochrome acridine orange (Sigma Aldrich, Missouri, USA) was dissolved in 1L of 0.1 M Phosphate Buffer Saline (PBS) and filtered through a 0.2 μl Sartorius filter. To make PBS, 8g of NaCl, 200mg of KCI, 1.44g of Na_2HPO_4, and 240mg of KH_2PO_4 (Univar, Illinois, USA) were dissolved in 800ml of distilled water. The pH was adjusted to the desired pH (7.4) and the PBS was autoclaved prior to the addition of the acridine orange. Following rinsing of SS biofilm coupons with distilled water, the coupons were fixed in a fixative (1% formalin) for 2 min. The fixed cells on the coupons were then immersed in the acridine orange solution for 2 min. The coupons were then gently rinsed with distilled water three times and air dried. Each coupon was mounted on a glass slide and examined under Olympus microscope B×53 with a FITC light excitation filter block using CellsSens Dimension software.

3.2.5. Scanning Electron Microscope (SEM)

The coupon samples were placed in primary fixative (Modified Karnovsky's fixative (3% gluteraldehyde 2% formaldehyde in 0.1M sodium cacodylate, pH 7.2) and the samples were fixed for at least 8 hours at room temperature. Following fixation, samples were washed three times (10-15 minutes each) in phosphate buffer (0.1M, pH 7.2) followed by dehydration in graded ethanol series (25%, 50%, 75%, 95%, 100%) for 15 minutes each and a final 100% ethanol wash for 1 hour. Samples were critical point dried using liquid CO_2 as the CP fluid and 100% ethanol as the intermediary (Polaron E3000 series II critical point drying apparatus).

Samples were mounted onto aluminium stubs using double sided tape and sputter coated with approximately 100nm of gold (Baltec SCD 050 sputter coater) and viewed in the FEI Quanta 200 Environmental Scanning Electron Microscope at an accelerating voltage of 20kV.

3.3. Statistical analysis and software

All data points were reported as means with standard deviations. The Plackett-Burman design for Section 3.1.2.1.was generated using Minitab 18. Data acquired were analysed with one-way analysis of variance (ANOVA, $p < 0.05$) on SPSS 18.0 and graphed using Origin 8.5 (Massachusetts, USA). For screening the enzymes using the Plackett-Burman design (Section 3.1.2.1.), Multivariate analysis of variance (MANOVA,$p < 0.05$) was used to examine the differences across multiple dependent variables simultaneously.

Chapter 4. Results

4.1. Selection of strains

Figure 6 below presents plate count results of the strains of the *A. flavithermus*, *B. licheniformis*, and *G. stearothermophilus* biofilms developed on SS coupons in a microtiter plate. All of the tested strains of the three species were able to form biofilms, but to a different extent. For *A. flavithermus,* the highest biofilm forming strain was T18C, followed by B12, P2, and T2. For *B. licheniformis*, strains C55C01 and C55C11were similar in the ability to form biofilms, and D55C11 was the least biofilm forming strain. For *G. stearothermophilus*, the best biofilm forming strain was identified to be P3, followed by D1 and A1. Therefore, strains B12 and T18C of *A. flavithermus*, C55C11 and C55C01 of *B. licheniformis*, and P3 and D1 of *G. stearothermophilus* were selected for further analysis.

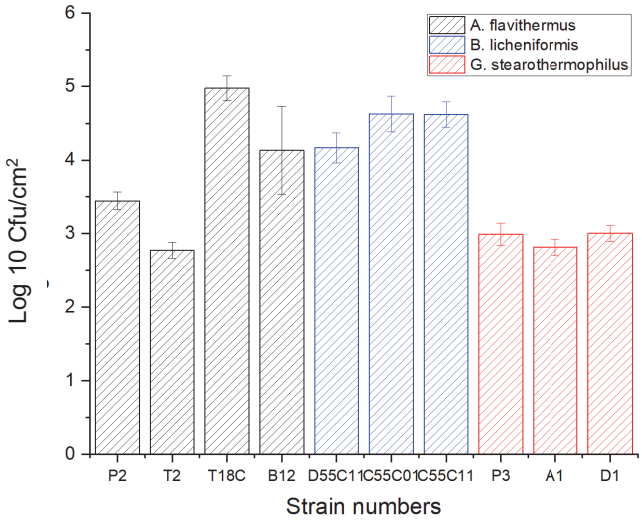


Figure 6. Plate counts of the strains of the thermophilic bacilli biofilms grown on SS in a microtiter plate. Error bars are standard deviations of triplicates of each strain

4.2. Screening enzymes for biofilm grown on SS coupons using Plackett-Burman design

Calibration equations (Table 5) were determined from BacTrac 4300 using impedance microbiology for strains B12 and T18C of *A. flavithermus*, strains C55C01 and C55C22 of *B. licheniformis*, and strains D1 and P3 of *G. stearothermophilus*. Based on the results of impedance detection for both M-value (3% threshold) and E-value (10% threshold), E-value growth curves were reproducible and applicable (i.e., curves derived from M-values displayed inconsistent and contradictory growth curves). Hence, the E-values were used in the study for establishing calibration curves and quantifying biofilm cells. The calibration curve equations

for all the strains of the thermophiles showed good correlations with R2>0.9. Conventionally, detection times obtained using impedance microbiology are converted into their equivalent plate count values in Log 10 CFU/ml based on the calibration curves. However, in this instance, when the BacTrac time results were converted into Log 10 CFU/ml, the results became negative values (i.e., slower growth of viable cells and metabolism) for some of the strains. Hence, for consistency of reporting the results, the BacTrac detection results for the present study were expressed in hours as opposed to their log counts counterpart.

Table 5. Calibration curves of the thermophiles using Impedance microbiology (BacTrac 4300)

	Strain	Calibration curve equation
A. *flavithermus*	B12	$y = -0.6272x + 6.1089$, $R^2 = 0.947$
	T18C	$y = -0.4933x + 5.6396$, $R^2 = 0.95$
B. licheniformis	C55C11	$y = -0.8237x + 7.7631$, $R^2 = 0.9828$
	C55C01	$y = -0.9403x + 8.172$, $R^2 = 0.9782$
G. stearothermophilus	D1	$y = -0.9287x + 7.7564$, $R^2 = 0.9924$
	P3	$y = -1.0344x + 7.2627$, $R^2 = 0.9743$

The biofilm cells of the strains B12 and T18C of *A. flavithermus* attached on the SS coupons in a microtiter plate were 4.3 and 3.5 log 10 CFU/cm2, and spores of 3.8 and 3 log 10 CFU/cm2. The total plate count of the control for T18C was 3 log 10 CFU/cm2 and the spores were 2.9 log 10 CFU/cm2. The total plate counts and spore counts for all enzymatic treatments and NaOH were below the detectable level. For a statistical analysis purpose, the plate counts with below the detectable level were entered as 1.17 log 10 CFU/cm2 (15 CFU/cm2). For both the strains, the BacTrac detection hours were the earliest for Before Cleaning (B.C), indicating the greatest biofilm cell numbers and contaminations, followed by Control treated with 85°C sterile water (C). The BacTrac detection hour of the enzymatic treatment combination 2 for T18C did not reach the 10% threshold of the E-value within 24 hours. For data analysis, BacTrac detection results that did not reach 10% threshold were recorded as 24 hours. Using MANOVA on SPSS 18 with $p < 0.05$, the enzymes that significantly reduced biofilm cell attachment based on impedance microbiology were identified to be amylase and protease (CB14057) for B12, and amylase for T18C.

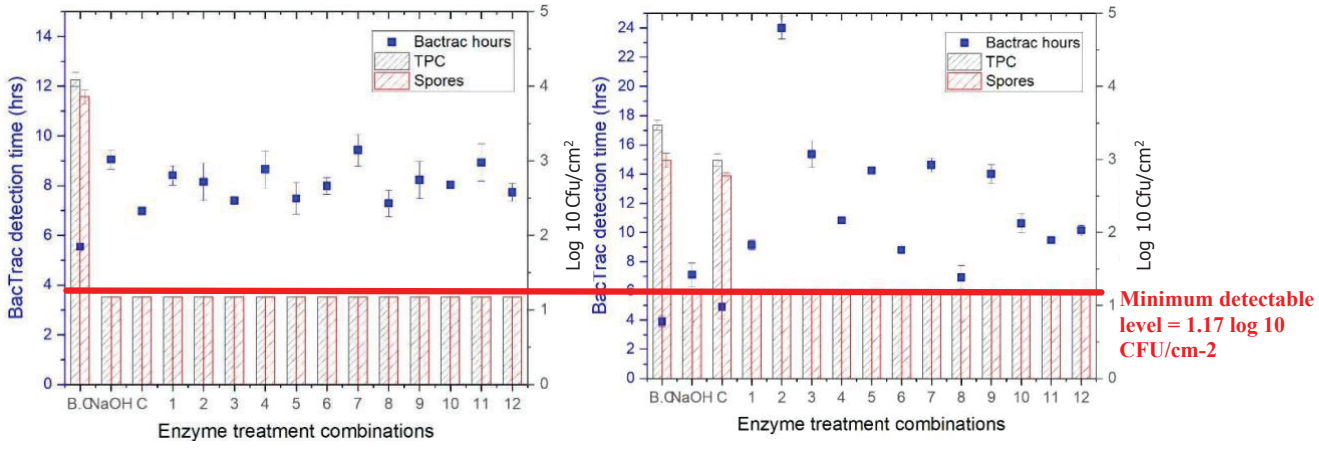


Figure 7. BacTrac hours, TPC and spore results of B12 (left) and T18C (right) of A. flavithermus using Plackett-Burman design

The biofilm cells of the strains P3 and D1 of *G. stearothermophilus* attached on the SS coupons in a microtiter plate were 4.8 and 4.6 log 10 CFU/cm^2, and spores of 4.5 and 4.2 log 10 CFU/cm^2. For both the strains, the BacTrac detection hours were the lowest for Before Cleaning (B.C), indicating the greatest biofilm cell numbers and contaminations. The plate and spore counts of NaOH for both the strains were below the minimum detectable level. The BacTrac detection hour of NaOH for P3 did not reach the 10% threshold of the E-value within 24 hours. Using MANOVA on SPSS 18 with $p < 0.05$, the enzymes that significantly reduced biofilm cell attachment determined from impedance microbiology were identified to be protease (CB14057) and endoglucanase (CB13961) for P3. No enzymes were found to be statistically significant in reducing the biofilm cells for strain D1.

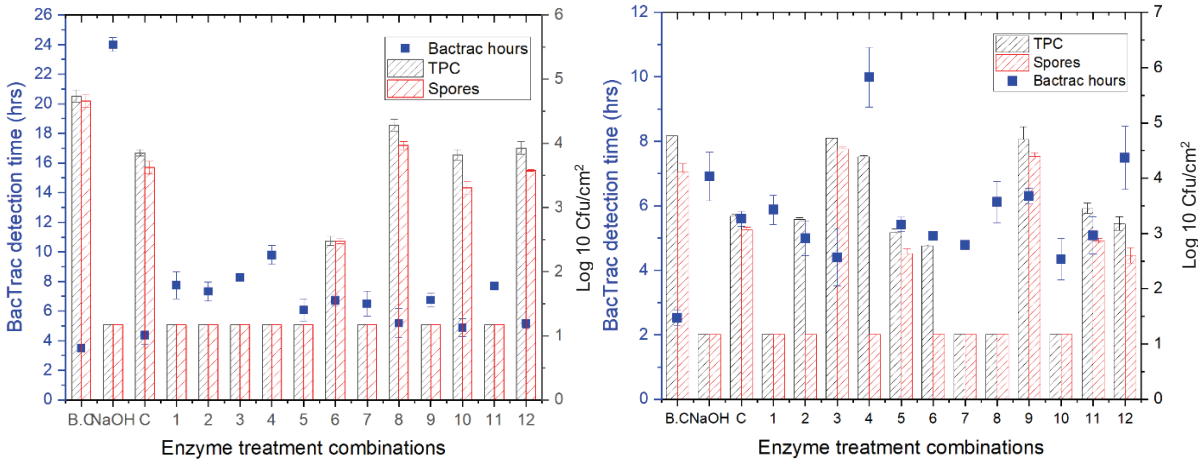


Figure 8. BacTrac hours, TPC and spore results of P3 (left) and D1 (right) of G. stearothermophilus using Plackett-Burman design

Coagulation of strain D1 (right) of *G. stearothermophilus* was compared with P3 (left) after 12 hour incubation in a microtiter plate. Notable milk coagulation was observed for D1 with a measured pH of 4.2, while P3 did not display prominent coagulations as also indicated by a higher pH (5.6). As a consequence, a considerable degree of fouling and adherence of vegetative cells developed for strain D1, which were difficult to rinse in the distilled water for bacterial enumeration.

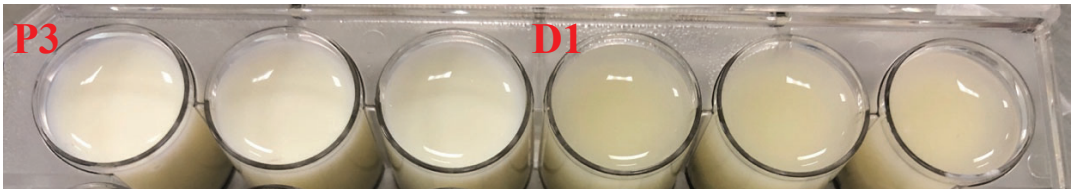


Figure 9. Pictures of P3 and D1 of G. stearothermophilus in a microtiter plate after incubation

The biofilm cells of the strains C55C01 and C55C11 of *B. licheniformis* attached on the SS coupons in a microtiter plate were 4.4 and 4.7 log 10 CFU/cm^2. No spores were observed for neither of the strains. The total plate counts and spore counts for all enzymatic treatments, as well as the control and NaOH were below the detectable level. For both the strains as anticipated, the BacTrac detection hours were the lowest (approximately 4.3 and 4.2 hours for C55C01 and C55C11 respectively). The BacTrac detection time for control of C55C01 was 9

hours. The BacTrac results for all the enzymatic treatments and NaOH for both the strains did not reach the 10% threshold of the E-value within 24 hours. No statistical analysis could be performed with the data obtained, indicating the specific enzymes that had the ability to decrease the adhered biofilm cells could not be defined. However, to further assess the effects of the enzymes on the biofilms developed in a biofilm reactor, amylase and protease (CB14057), which were found to be effective for the other thermophiles tested, were examined on the strains of *B. licheniformis*.

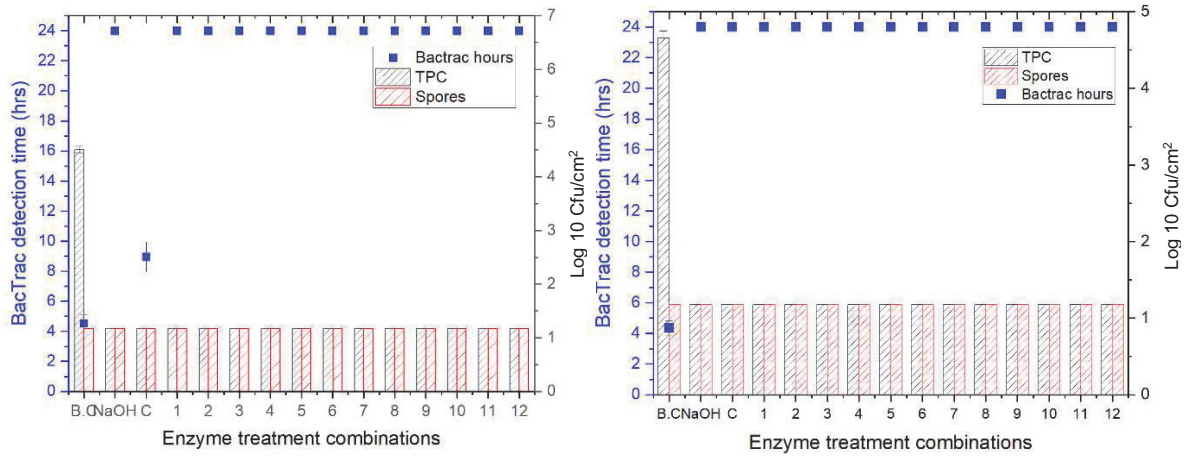


Figure 10. BacTrac hours, TPC and spore results of C55C01 (left) and C55C11 (right) of B. licheniformis using Plackett-Burman design

Table 6 below summarises the enzymes that significantly ($p<0.05$) decreased the attached biofilm cells of *A. flavithermus, B. licheniformis,* and *G. stearothermophilus* developed on SS coupons in a microtiter plate.

Table 6. Summary table of the enzymes shown to reduce the thermophilic bacilli biofilm cells using Plackett-Burman design

	Strain	Enzymes reducing biofilm	Assay Method
A. *flavithermus*	B12	Amylase and Protease (CB14057)	BacTrac hours
	T18C	Amylase	BacTrac hours
B. licheniformis	C55C11	Cannot be determined	-
	C55C01	Cannot be determined	-
	D1	None	None
G. stearothermophilus	P3	Protease (CB14057) and Endoglucanase (CB13961)	TPC and spore count

4.3. Quantification of biofilm cells grown on SS coupons in a CDC biofilm reactor in a batch mode

The enzymes that significantly reduced the attached biofilm cells in Section 4.2. were further investigated in a CDC biofilm reactor under a batch system. For convenience, protease CB14057 and endoglucanase CB13961 used in the studies onwards will be denoted as protease and endoglucanase. As Figure 11 illustrates, the plate count and spores of untreated coupons (Before Cleaning (B.C)) of T18C were the greatest, followed by control. The plate count and spores of NaOH and amylase were below the detection limit. Correspondingly, the BacTrac detection hour was the lowest for Before Cleaning, control, then NaOH and Amylase equally, signifying the least degree of contamination and presence of metabolising cells. The plate count and spores of B12 were significantly higher for B.C, and those for the enzymatic treatments as well as NaOH and control were below the detection limit. Likewise, the BacTrac detection time for B.C was the lowest, implying the greatest bacterial growth in the medium, followed by control. The detection hours of NaOH, amylase, protease and amylase + protease were within the similar range, being no significantly different to one another.

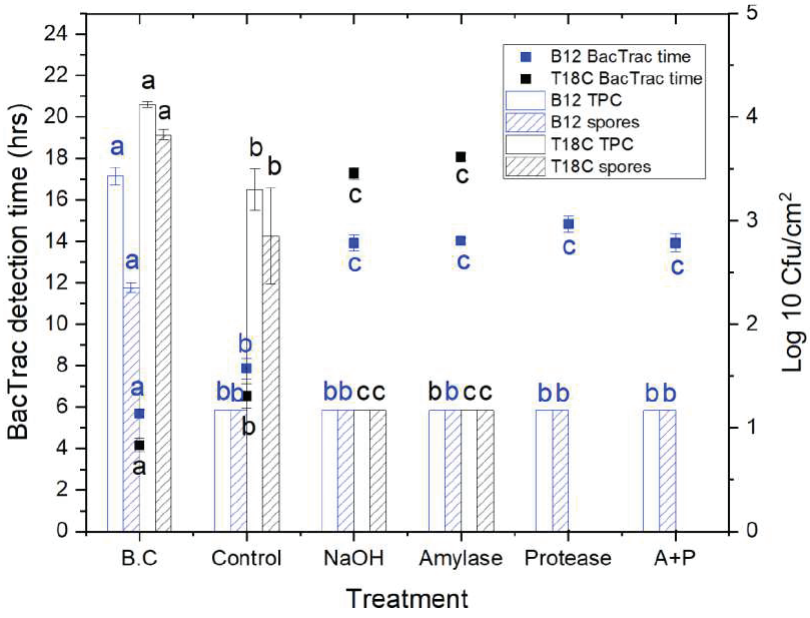
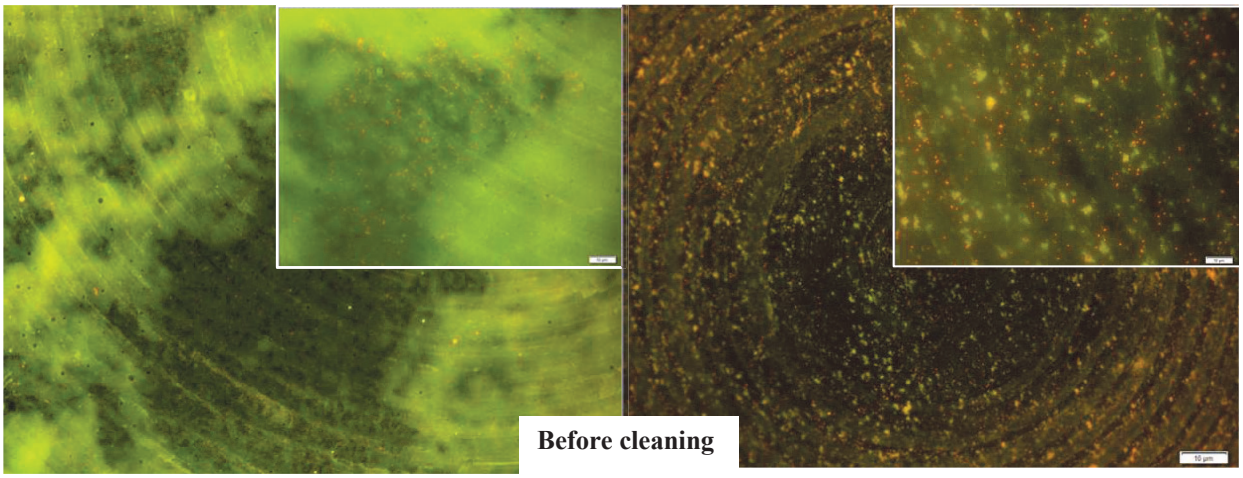
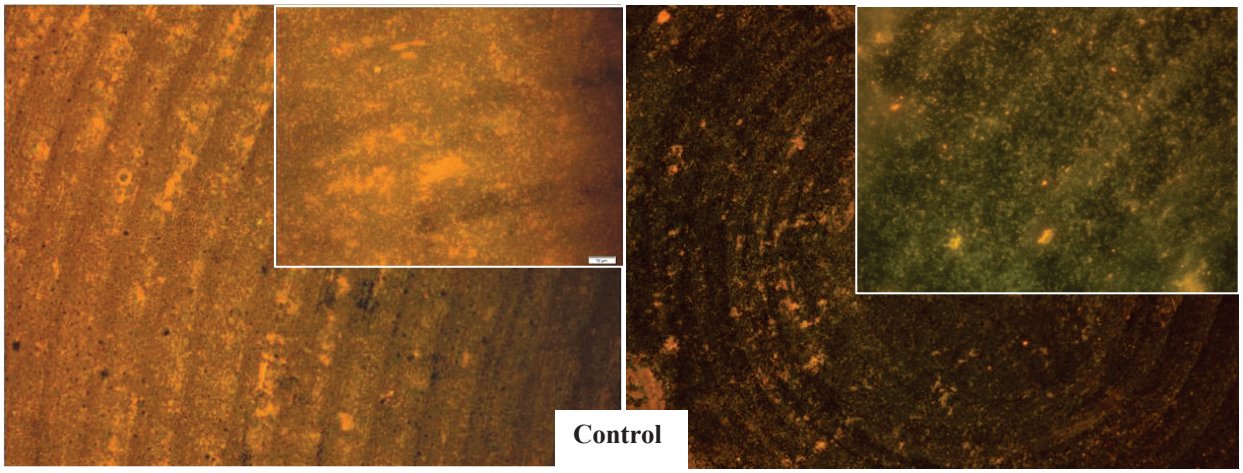


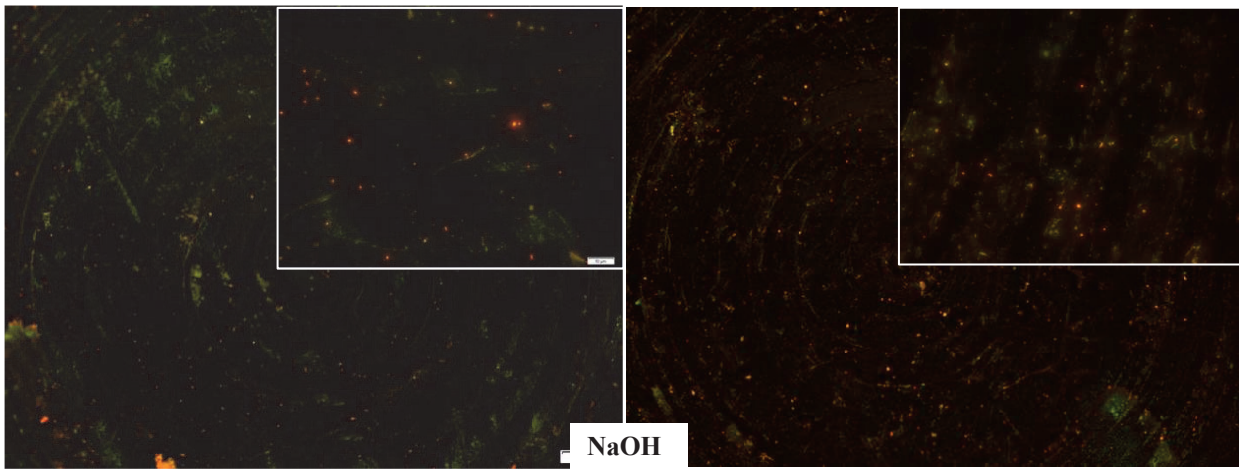
Figure 11. BacTrac hours, TPC and spore results of A. flavithermus grown in a CDC biofilm reactor in a batch system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test and error bars are standard deviations of triplicates of each strain

For morphological examinations of the attached biofilm cells in addition to viable cell analysis using plate count and impedance microbiology, epifluorescence microscopy using acridine orange was used. Based on three areas observed on the coupons, B.C of both strains T18C and B12 of *A. flavithermus* displayed EPS and bacterial cells, each indicated by green and orange fluorescence. Concerning the presence of EPS on the surface, the Control images of both the strains exhibited a similar pattern, without a notable difference from Before Cleaning. A significant decrease in EPS and bacterial cells was noted for NaOH. Amylase also showed substantial cleaning efficacy, relatively more on EPS than bacterial cells in comparison to NaOH, which was capable of removing both the EPS and bacterial cells.

Protease treatment on strain B12 biofilm was not shown to be significantly efficient, with much EPS and residues still remaining on the surface. The enzymatic treatment of the combination of amylase and protease was similar to that of the amylase treatment on its own, along with some EPS still attached.







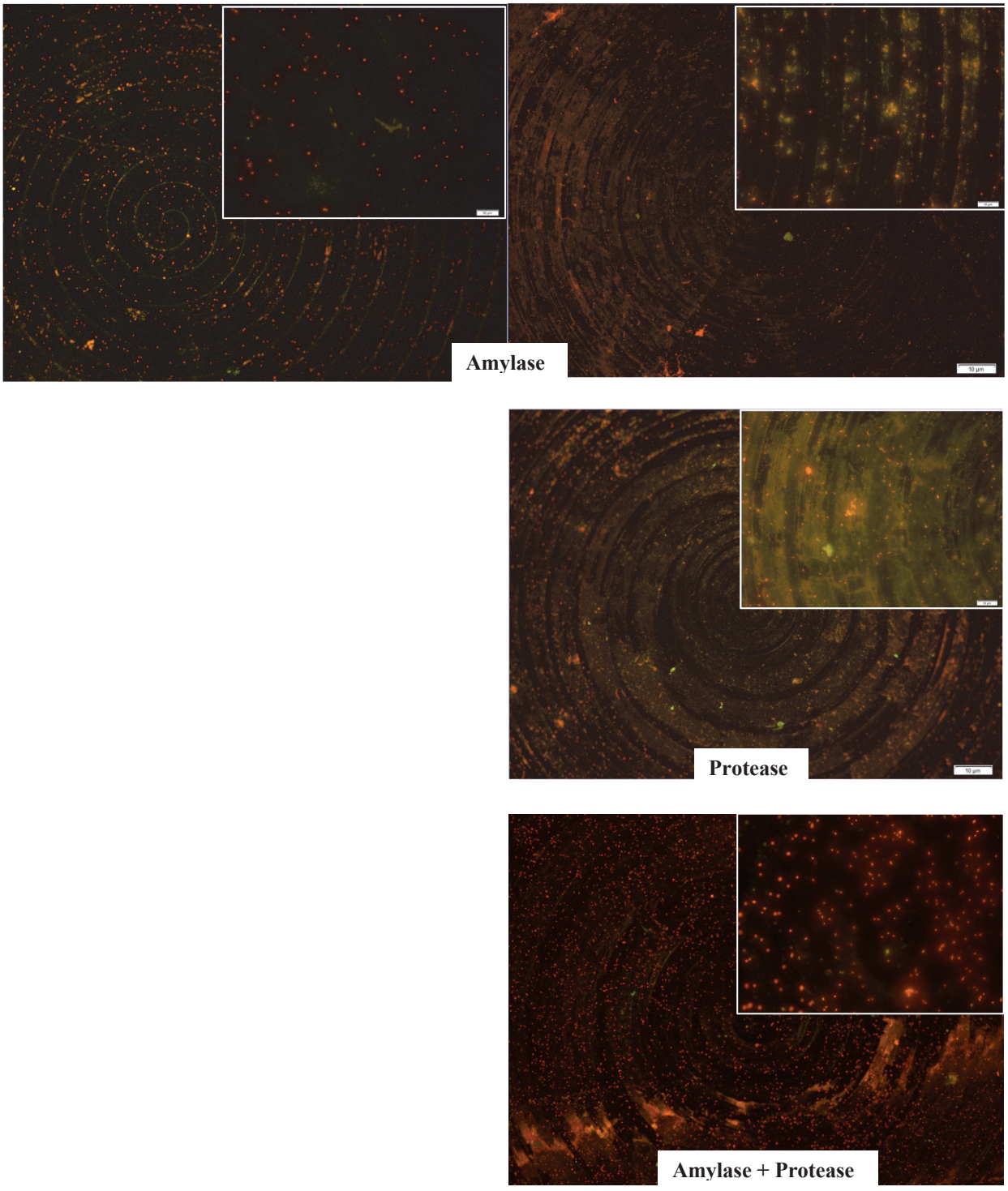


Figure 12. Images of the biofilms of T18C (left) and B12 (right) of A. flavithermus grown in a CDC biofilm reactor in a batch system under epifluorescence microscopy using acridine orange. The big image is 10um and the small image is 4um.

No spores were identified for both the strains of *B. licheniformis* (Figure 13). The plate counts were significantly higher than control, NaOH and the enzymatic treatments, and no statistical difference existed amongst them. In the same vein, the BacTrac detection hour was the lowest for C55C01 with the greatest bacterial growth, and the detection times for control,

NaOH and the enzymatic treatments did not reach the 10% threshold within 24 hours. The BacTrac detection time was the lowest for strain C55C11, then control, which was significantly lower than those of NaOH, amylase, protease and amylase + protease.

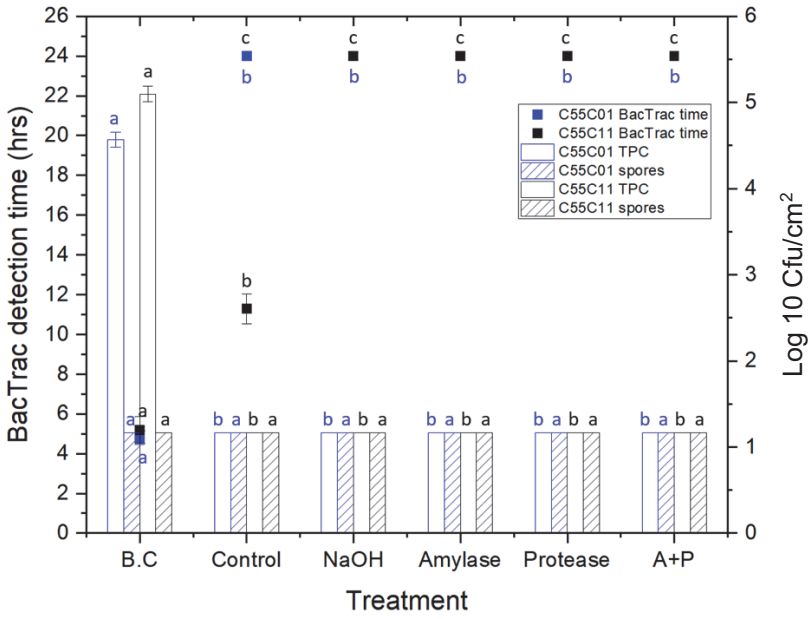
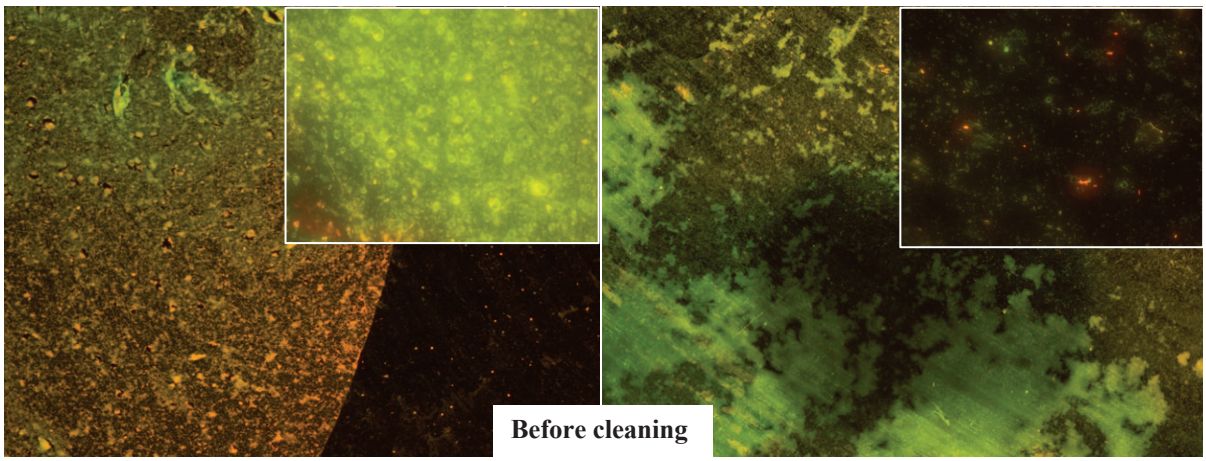
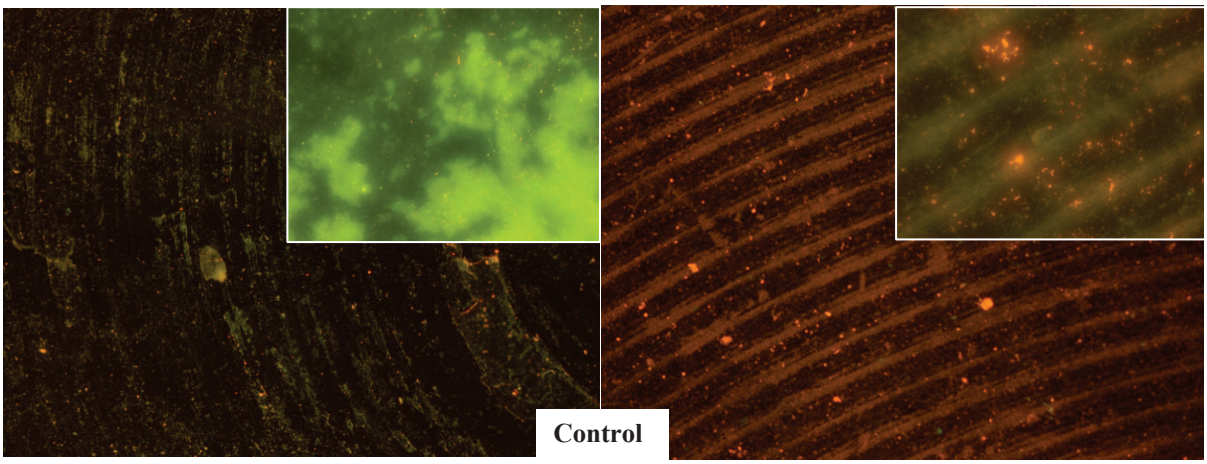
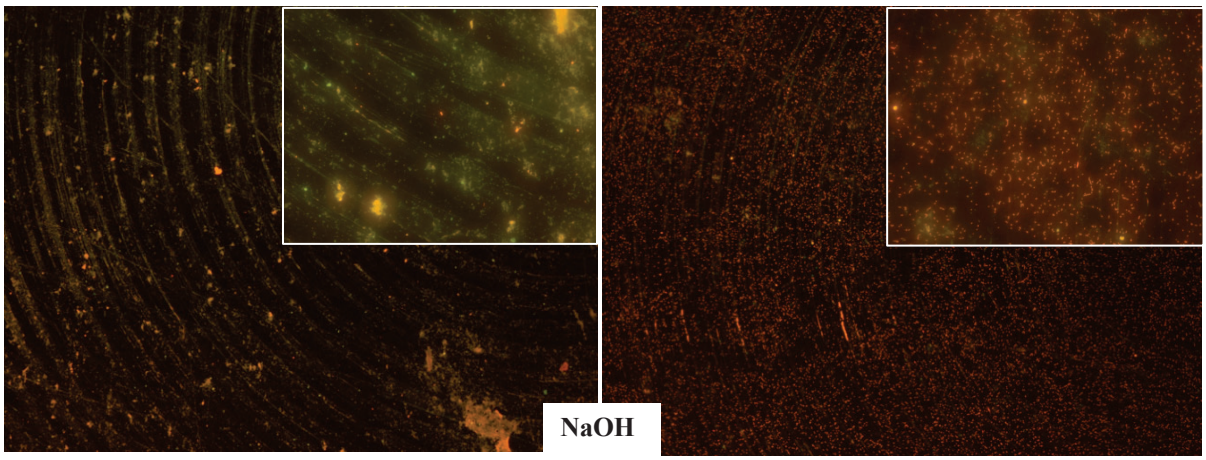


Figure 13. BacTrac hours, TPC and spore results of B. licheniformis grown in a CDC biofilm reactor in a batch system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test and error bars are standard deviations of triplicates of each strain

Distinguished EPS (indicated by green fluorescence) was observed for Before Cleaning for both the strains of *B. licheniformis.* Reduction in EPS and cells was noticed for NaOH, yet many residues still remained on the surface to a certain degree. Amylase was shown to have a substantial cleaning capability on reducing both EPS and bacterial cells, which differed from *A. flavithermus,* where it was observed amylase was more efficient in removing EPS than cells. Protease presented the least efficacy in cleaning the coupon surfaces shown by the large amount of EPS and cells still adhered following the cleaning practice. As anticipated, fluorescence images of the enzyme treatment of the combination (amylase and protease) were similar to those of amylase and protease treatments on their own.


Before cleaning


Control


NaOH

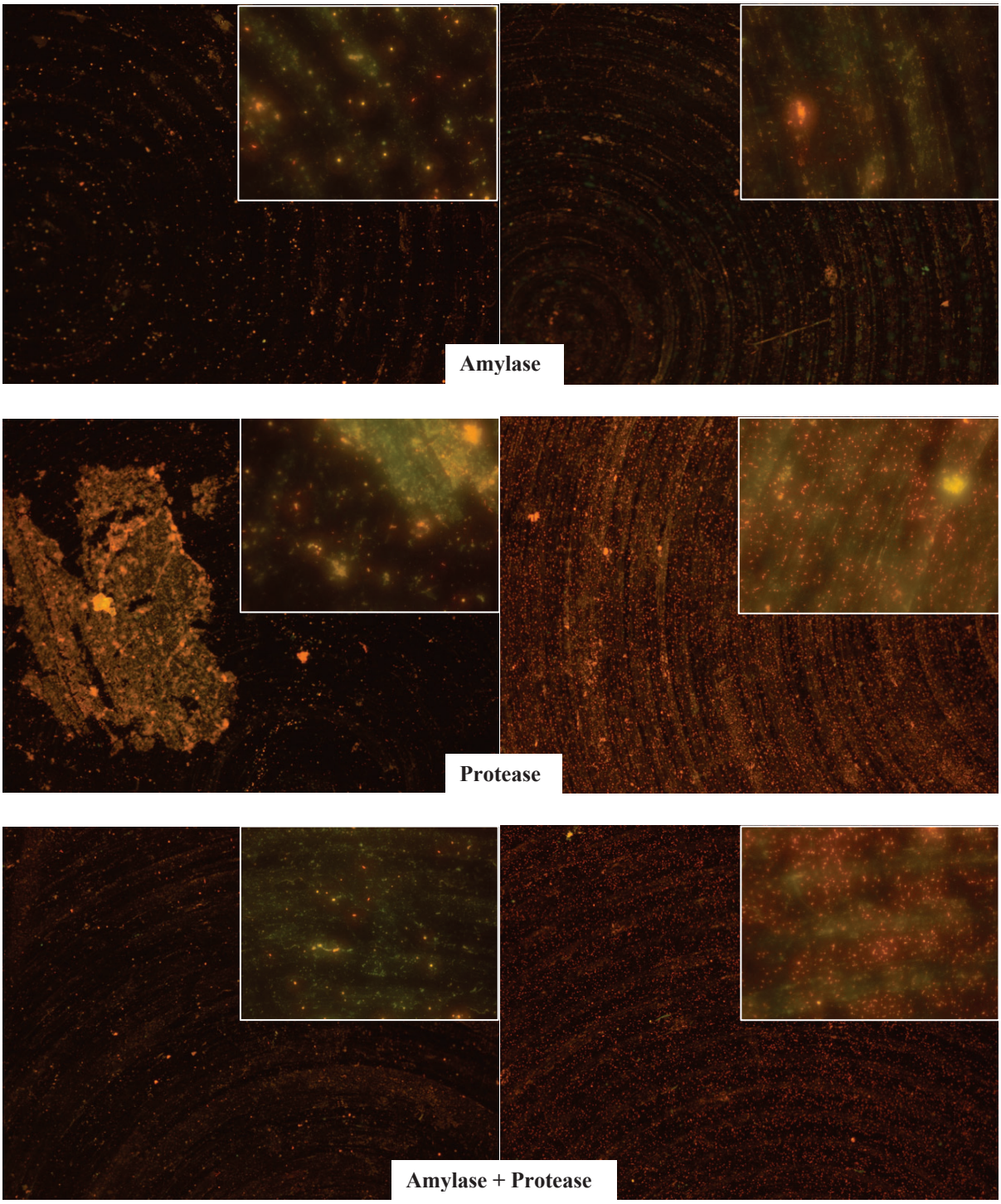


Figure 14.Images of the biofilms of C55C01 (left) and C55C11 (right) of B. licheniformis grown in a CDC biofilm reactor in a batch system under epifluorescence microscopy using acridine orange. The big image is 10um and the small image is 4um.

The plate count of B.C of strain P3 of *G. stearothermophilus* was the greatest, followed by control. The plate counts of treatments of endoglucase, protease and endoglucanase + protease were below the detection limit. B.C had the greatest spores, while the enzymatic treatments, control and NaOH had spores below the detection limit. The BacTrac detection hour was the lowest for B.C, then control. The detection times for NaOH and amylase were significantly higher than B.C (a), control (b), and protease and endoglucanase+ protease (d).

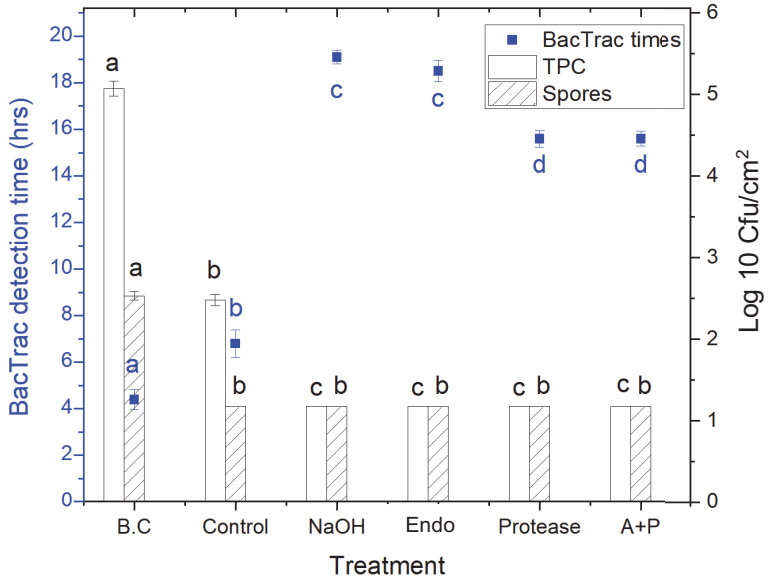


Figure 15. BacTrac hours, TPC and spore results of strain P3 of G. stearothermophilus grown in a CDC biofilm reactor in a batch system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test and error bars are standard deviations of triplicates of each strain.

As previously observed for *A. flavithermus* and *B. licheniformis,* the most EPS and bacterial cells were observed for Before Cleaning, followed by control. For NaOH, a large number of cells survived the treatment on the surface similar to control, however, the majority of the cells were strained orange, indicating dead cells. Further in line with the prior examinations, endoglucanase exhibited a similar behaviour to amylase with an enhanced ability to remove EPS than bacterial cells. Protease also performed in a close way to *A. flavithermus* and *B. licheniformis*, where it showed less efficacy in eliminating the EPS of the biofilm.

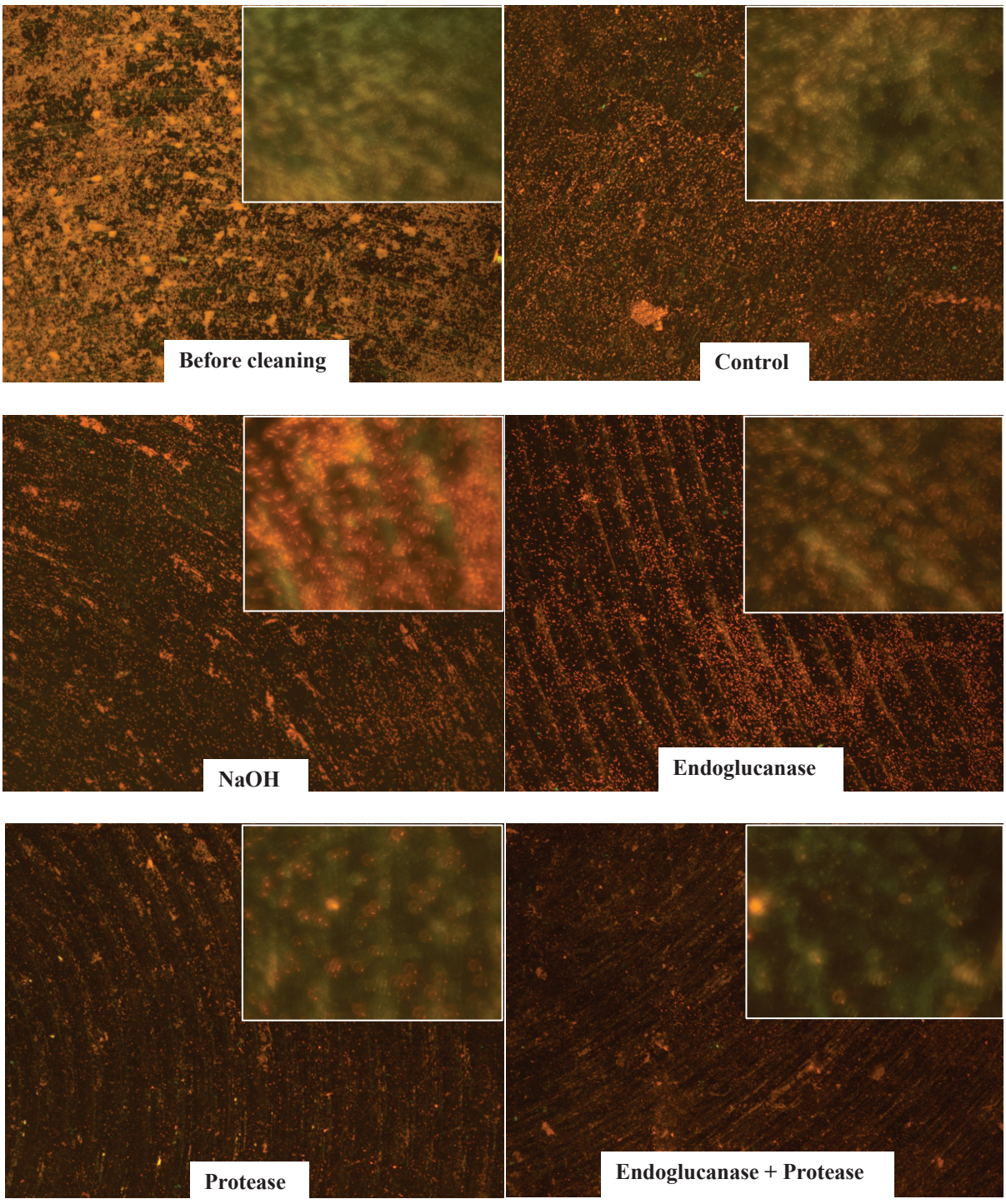


Figure 16. Images of the biofilms of P3 of G. stearothermophilus grown in a CDC biofilm reactor in a batch system under epifluorescence microscopy using acridine orange. The big image is 10um and the small image is 4um.

4.4. Evaluation of the efficiency of the screened enzymes on biofilms grown in a continuous flow biofilm reactor

To determine the doubling times and flow rates of the strains required for experimenting the continuous flow reactor, growth curves were initially established using T18C of *A. flavithermus*, C55C11 of *B. licheniformis*, and P3 of *G. stearothermophilus* in batch reactors (Figure 17). With the starting bacterial concentrations ranging from 10^4 to 10^5, the exponential phase was reached within 24 hours of the sampling time. *B. licheniformis,* both mesophilic and thermophilic, obtained a gradual incline, while the two thermophilic species displayed steeper exponential growth.

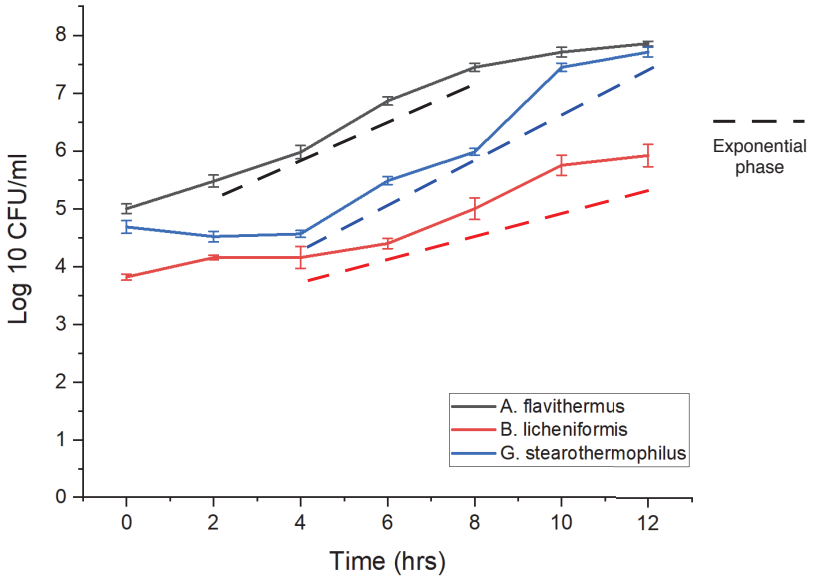


Figure 17. Growth curves of the thermophiles grown in the CDC biofilm reactor under a batch system. Error bars are standard deviations of triplicates of each strain

Based on the exponential phase from the growth curves acquired, doubling times and flow rates were calculated as shown in Table 7. As also previously revealed in Figure 17, the results were as anticipated, with *B. licheniformis* having the longest doubling time (53 mins), followed by *A. flavithermus* (49 mins), and *G. stearothermophilus* (38 mins). Hence, the flow rates were determined to be 0.82, 0.89, and 0.63 ml/min for *A. flavithermus, B. licheniformis,* and *G. stearothermophilus* respectively.

Table 7. Doubling times and flow rates calculated from the growth curves

	Doubling time (min)	Flow rate (ml/min)
A. flavithermus	49.17	0.82
B. licheniformis	53.30	0.89
G. stearothermophilus	37.59	0.63

A strain was selected for each *A. flavithermus*, *B. licheniformis,* and *G. stearothermophilus* to be examined in a continuous flow reactor. The plate count and spores of Before Cleaning were significantly higher than those of control, and NaOH and amylase which were below the detection limit. Accordingly, the BacTrac detection hour was the lowest for Before Cleaning, and NaOH and Amylase detection times did not reach the 10% threshold value within the 24 hours of the examination time.

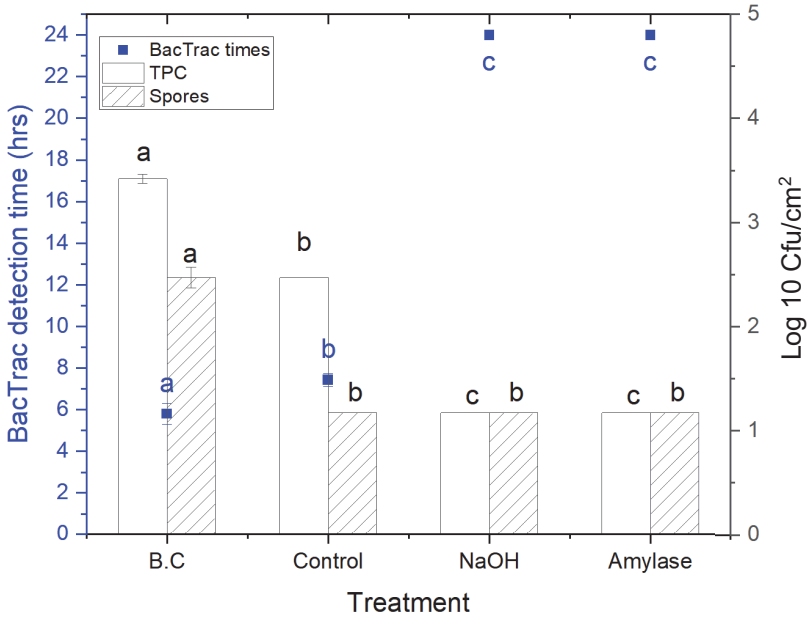
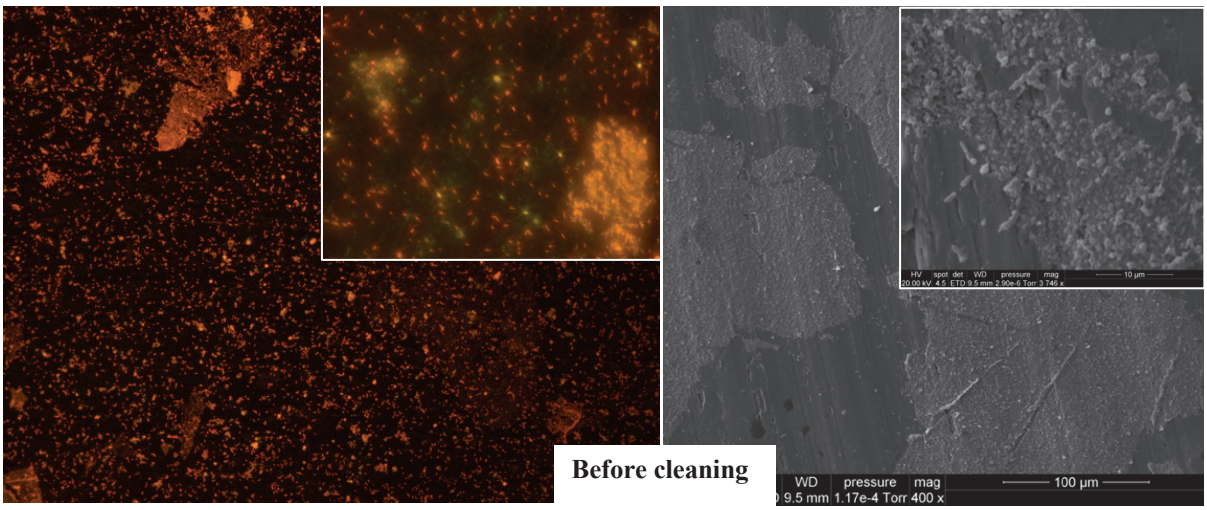
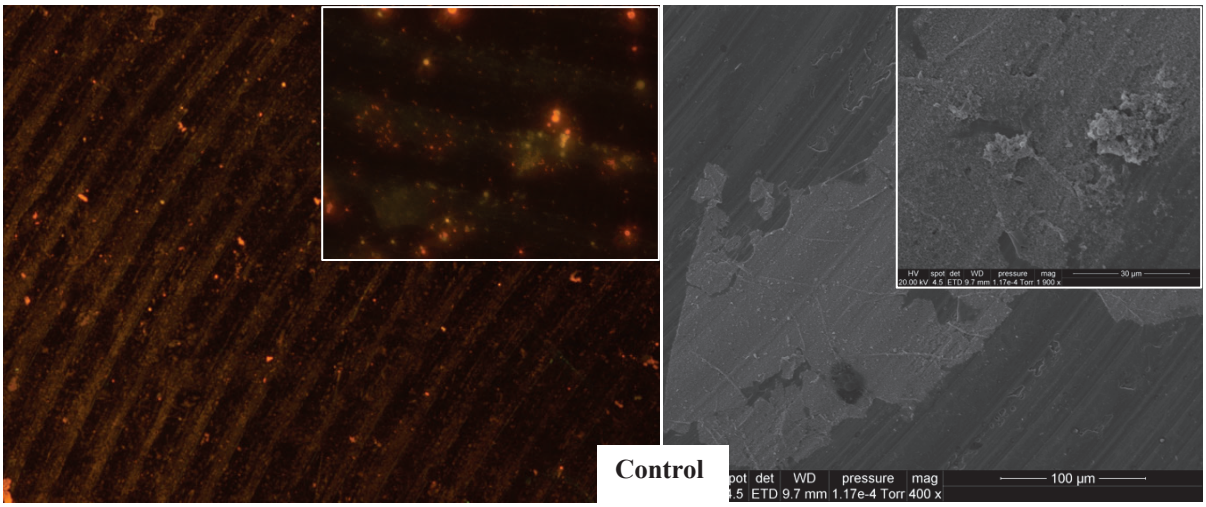
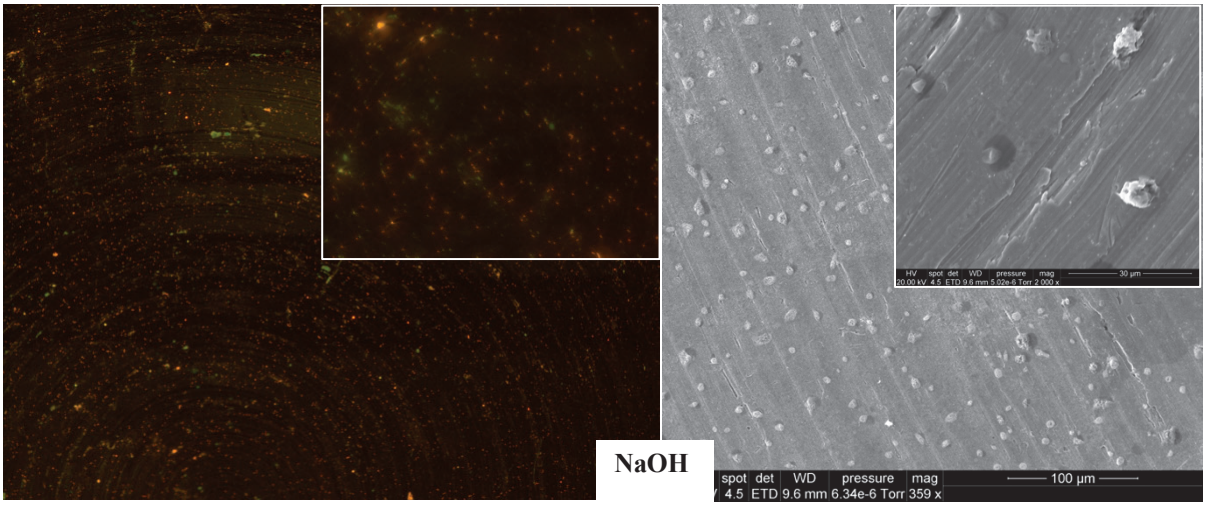


Figure 18. BacTrac hours, TPC and spore results of strain T18C of A. flavithermus grown in a CDC biofilm reactor in a continuous flow system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test and error bars are standard deviations of triplicates of each strain.

For further assessments of the morphological characteristics and compositions of the biofilm matrices, Scanning Electron Microscopy (SEM) was used in addition to the epifluorescence microscopy. The epifluorescence microscopy images and SEM images were in correspondence with each other. While images of both Before Cleaning and control display agglomerated biofilm cells and EPS remained attached on the surfaces, NaOH showed substantially less cells and EPS in the microscopy images. For NaOH, the majority of the substances attached on the surface were presumed to be protein residues. Amylase images also align with the previous observations, where the treatment was shown to be more efficient on removing the EPS than bacterial cells. The SEM images of amylase show clustered bacterial cells still present on the surface.


Before cleaning
WD pressure mag 100 µm
9.5 mm 1.17e-4 Torr 400 x


Control
spot det WD pressure mag 100 µm
4.5 ETD 9.7 mm 1.17e-4 Torr 400 x


NaOH
spot det WD pressure mag 100 µm
4.5 ETD 9.6 mm 6.34e-6 Torr 359 x

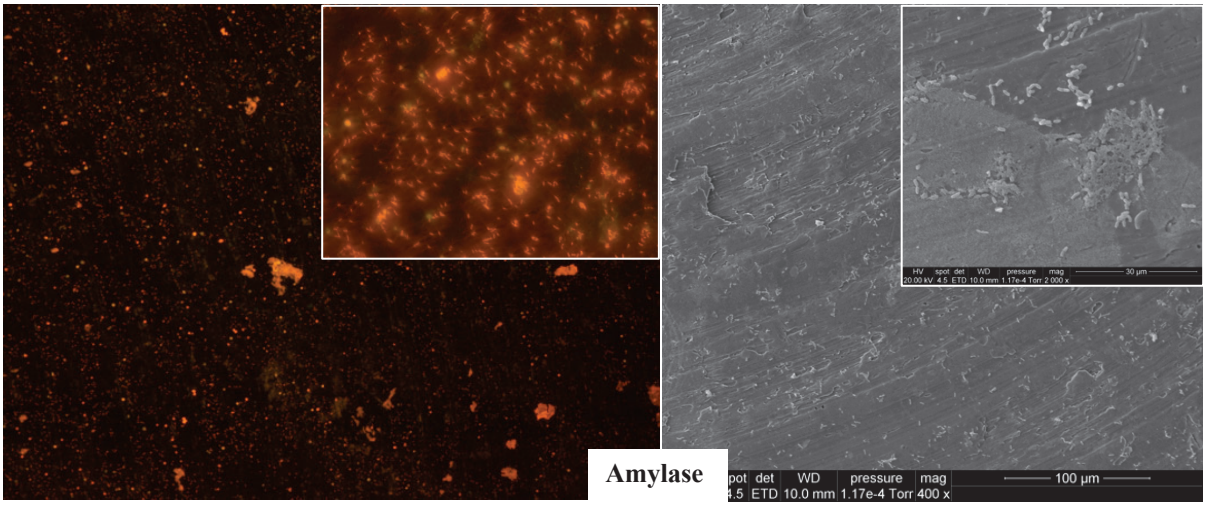


Figure 19. Images of the biofilms of T18C of A. flavithermus grown in a CDC biofilm reactor in a continuous flow system under epifluorescence microscopy using acridine orange (left). The big image is 10um and the small image is 4um. Images on the right are SEM images with scales shown.

Strain C55C11 of *B. licheniformis* showed closely aligned results with the previous examinations, where Before Cleaning had the highest contamination based on both plate and spore counts, and BacTrac detection times. The plate count and spores of NaOH and the enzyme treatments were below the detection limit, and the BacTrac detection times did not reach the set threshold within the 24 hours.

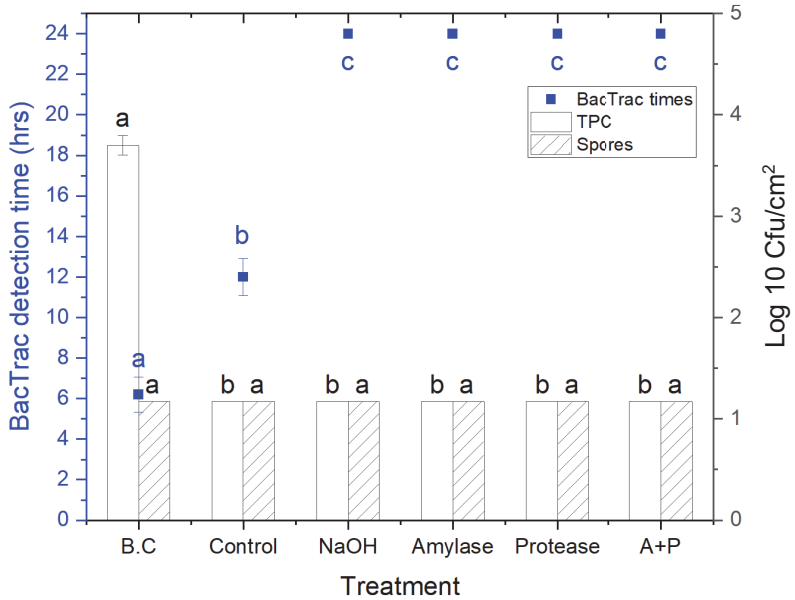
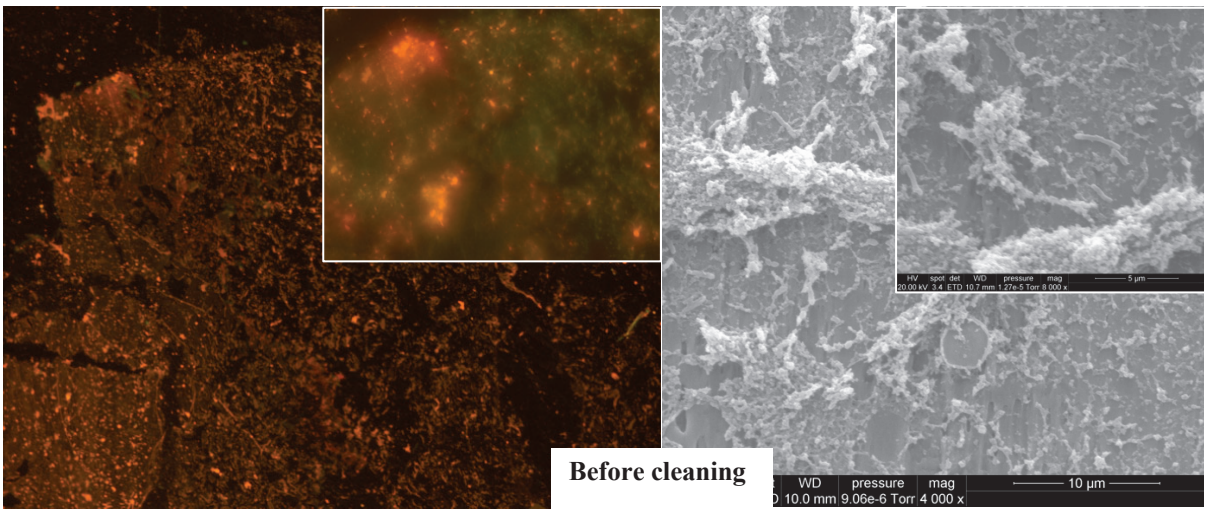
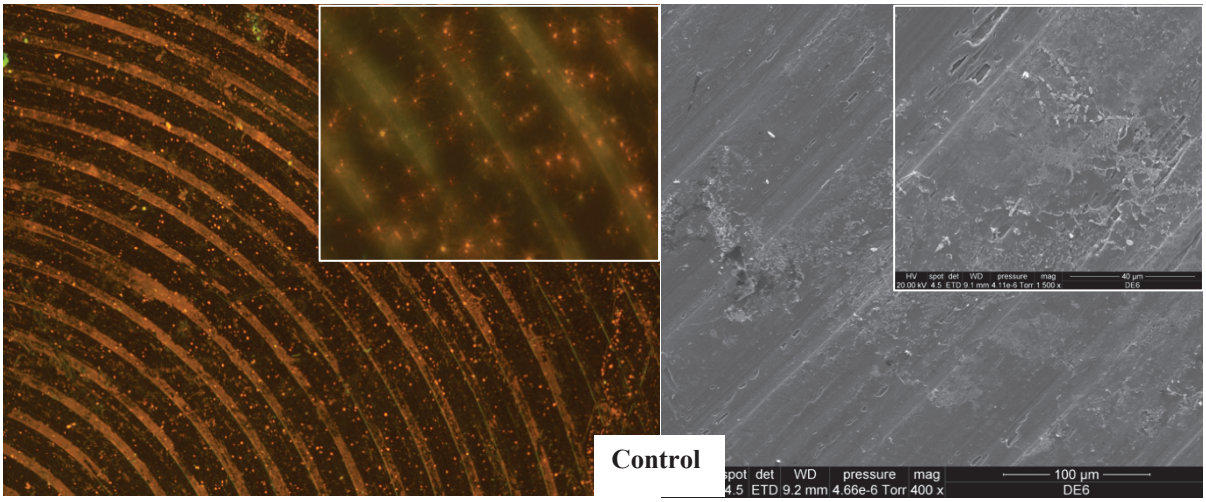
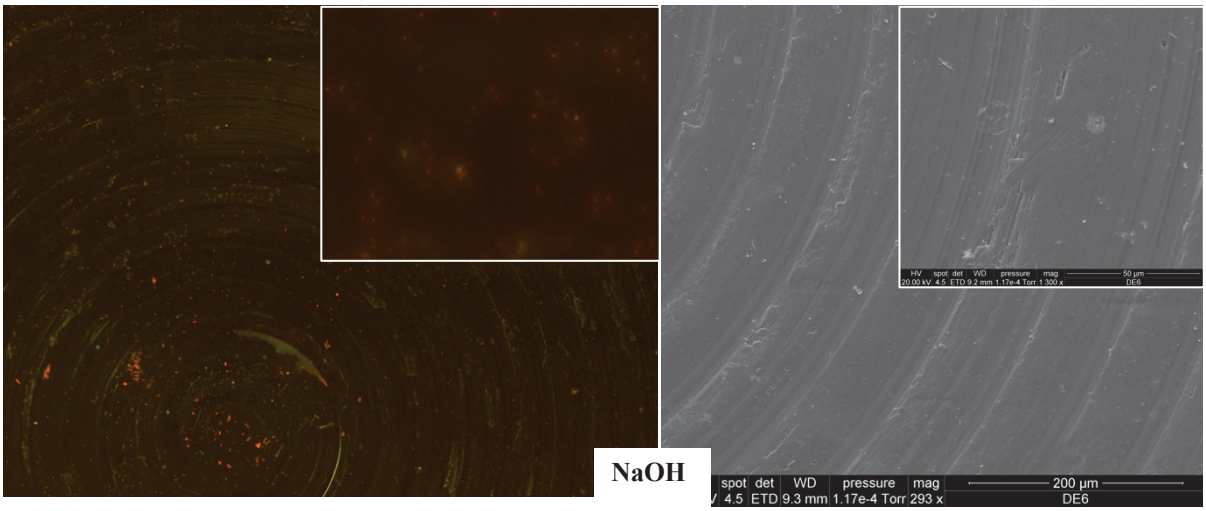


Figure 20. BacTrac hours, TPC and spore results of strain C55C11 of B. licheniformis grown in a CDC biofilm reactor in a continuous flow system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test and error bars are standard deviations of triplicates of each strain.

The epifluorescence microscopy and SEM images show a significant reduction on the viable cells and biofilm EPS on the surfaces treated with NaOH, which is in agreement with those from the batch reactor system. Similarly, amylase treatment had fewer cells, and presumably protein residues existed on the surface. Protease also had a vast amount of EPS remaining on the surface following treatment.


Before cleaning
WD pressure mag
10.0 mm 9.06e-6 Torr 4 000 x
10 µm


Control
spot det WD pressure mag
4.5 ETD 9.2 mm 4.66e-6 Torr 400 x
100 µm
DE6


NaOH
spot det WD pressure mag
4.5 ETD 9.3 mm 1.17e-4 Torr 293 x
200 µm
DE6

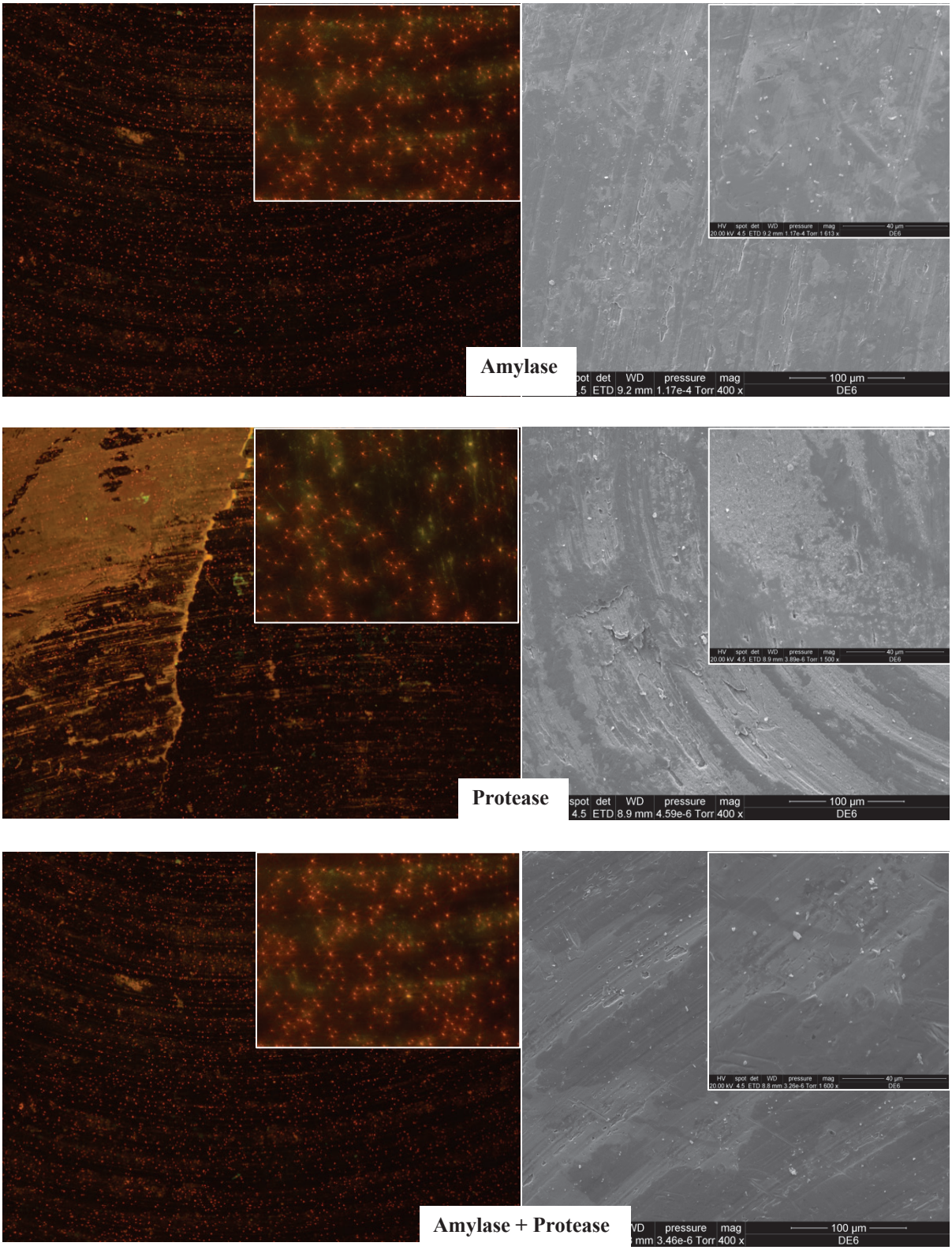


Figure 21. Images of the biofilms of C55C11 of B. licheniformis grown in a CDC biofilm reactor in a continuous flow system under epifluorescence microscopy using acridine orange (left). The big image is 10um and the small image is 4um. Images on the right are SEM images with scales shown.

The plate count and spores of control, NaOH and the enzymatic treatments were all below the detection limit. The BacTrac detection hours were also in accordance with the standard plate count results, with those for Before Cleaning and control being significantly lower than the rest, indicating the greatest contamination. The BacTrac time for NaOH did not reach the threshold within 24 hours, and endoglucanase had an equally high BacTrac detection time, followed by endoglucanase + protease treatment, and protease.

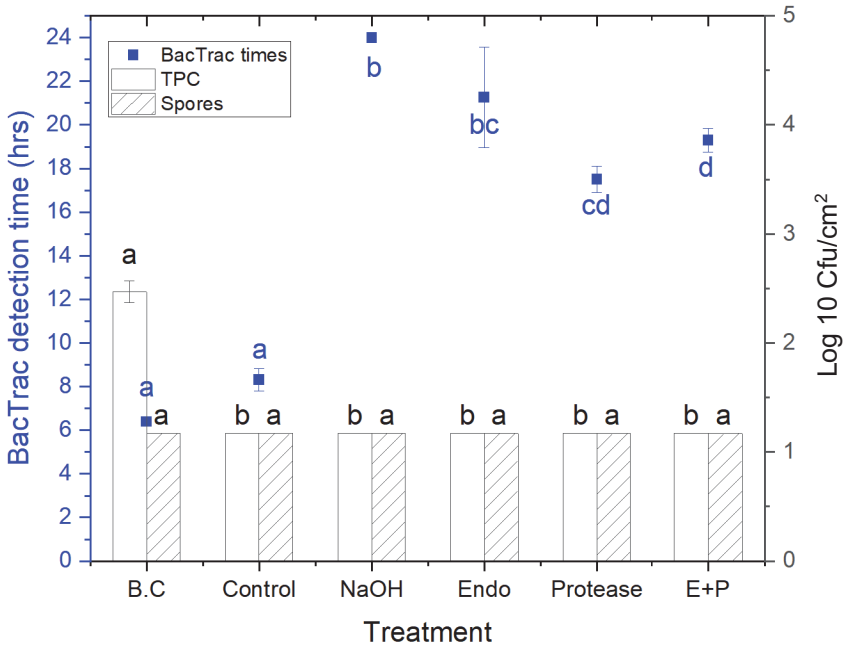
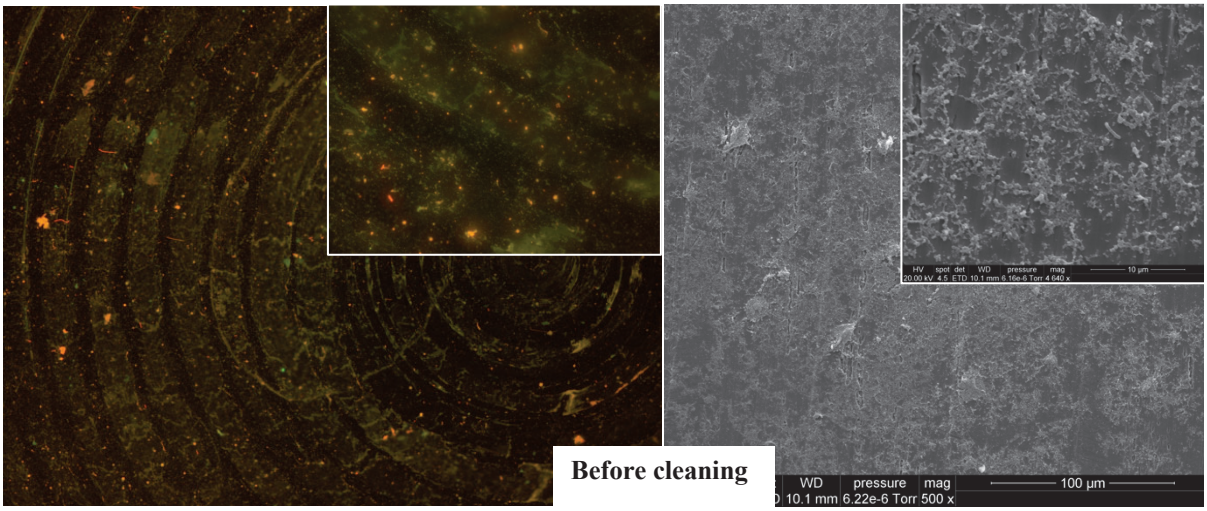
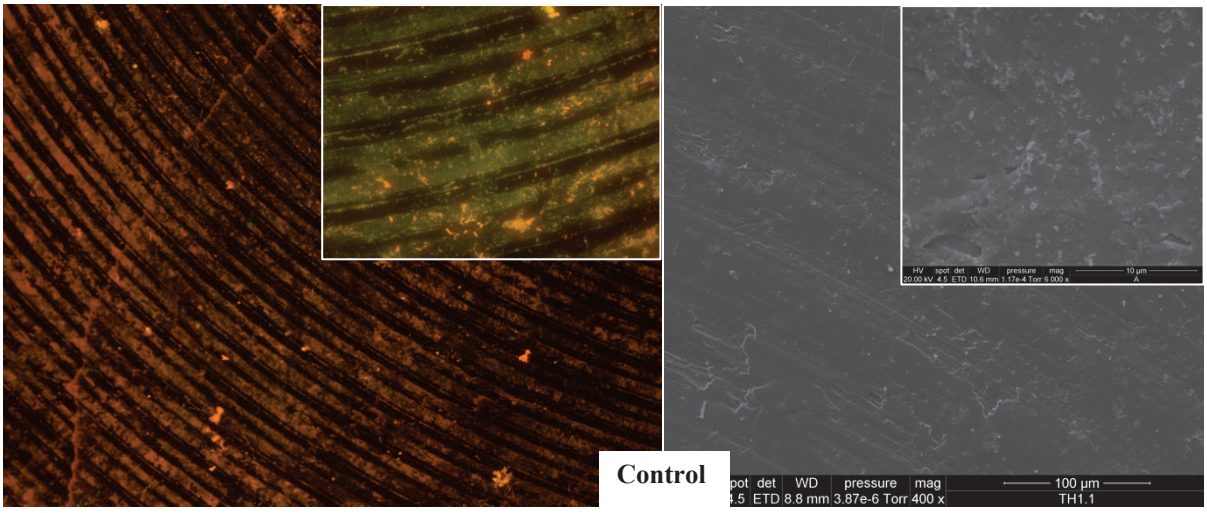
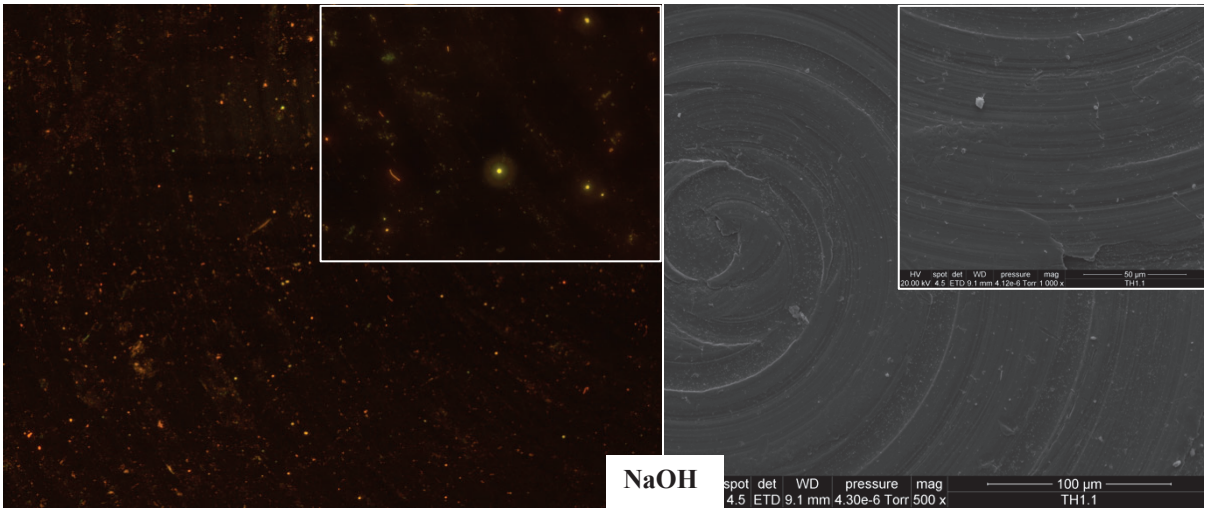


Figure 22. BacTrac hours, TPC and spore results of strain P3 of G. stearothermophilus grown in a CDC biofilm reactor in a continuous flow system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test and error bars are standard deviations of triplicates of each strain.

The SEM images for the control of strain P3 of *G. stearothermophilus* displayed a considerable amount of residues left on the surface. The NaOH showed a distinguishable reduction in viable biofilm cells and EPS, and endoglucanase also demonstrated a decrease in the cells and EPS. As previously observed for protease, the epifluorescence microscopy and SEM images exhibited a certain degree of remaining EPS and cells.


Before cleaning
WD pressure mag 100 µm
10.1 mm 6.22e-6 Torr 500 x


Control
pot det WD pressure mag 100 µm
.5 ETD 8.8 mm 3.87e-6 Torr 400 x TH1.1


NaOH
spot det WD pressure mag 100 µm
4.5 ETD 9.1 mm 4.30e-6 Torr 500 x TH1.1

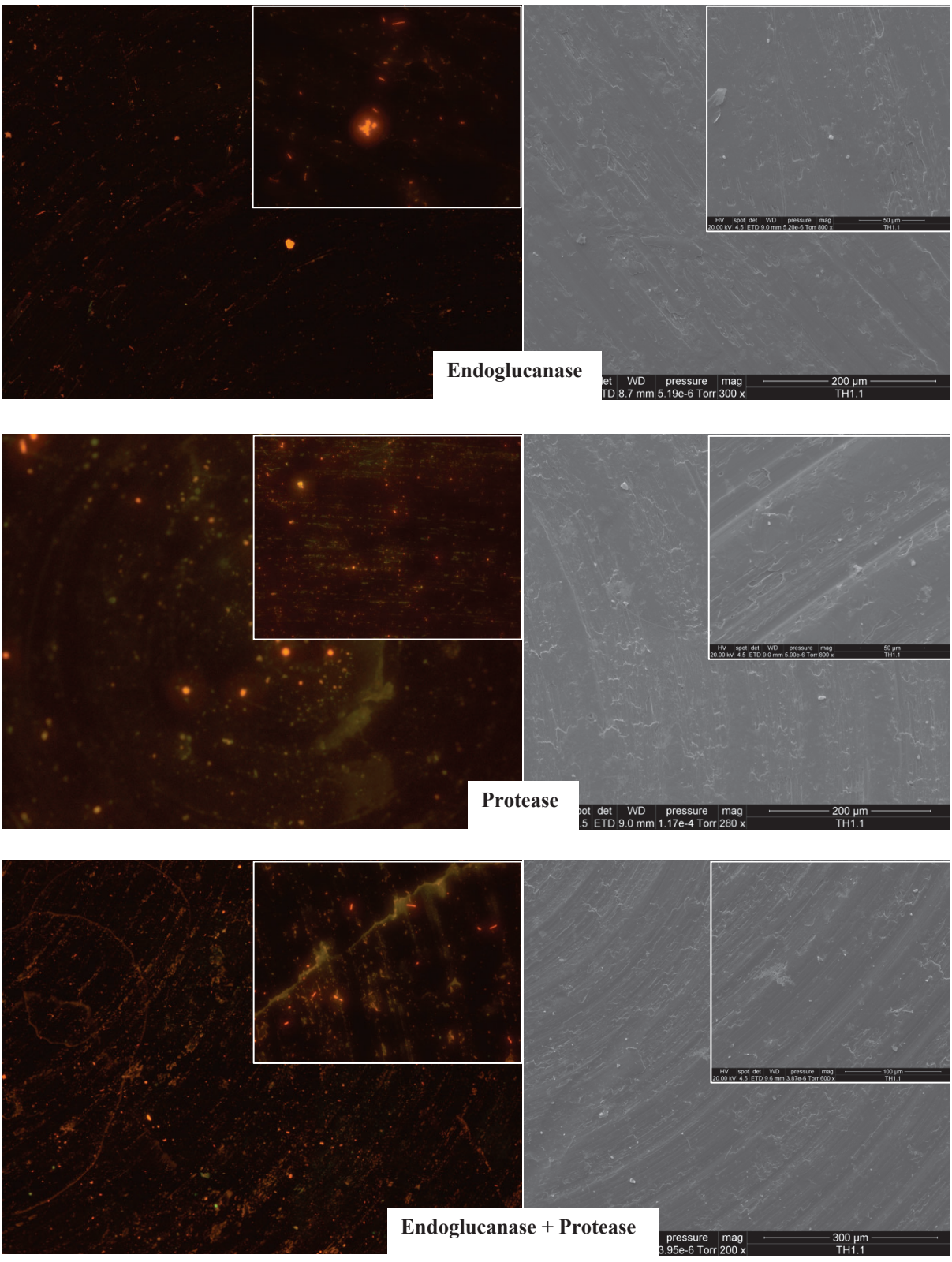


Figure 23. Images of the biofilms of P3 of G. stearothermophilus grown in a CDC biofilm reactor in a continuous flow system under epifluorescence microscopy using acridine orange (left). The big image is 10um and the small image is 4um. Images on the right are SEM images with scales shown.

The biofilm formations of T18C of *A. flavithermus*, C55C11 of *B. licheniformis* and P3 of G. *stearothermophilus* under different growth systems were compared and evaluated. The biofilm incubation time was increased from 12 hours in the microtiter plate to 24 hours in the CDC biofilm reactors (both batch and continuous flow systems) for greater bacterial counts in an attempt to demonstrate higher log reductions by the enzymatic treatments. The impedance microbiology results suggest that the BacTrac detection times for CDC biofilm reactor under a continuous flow system were significantly longer than those of the batch system, and in the microtiter plate. Similarly, the finding was also reflected by the standard plate count method, where the plate count results for the CDC biofilm reactor under a continuous flow system were significantly lower than those of the batch system and microtiter plate cell counts.

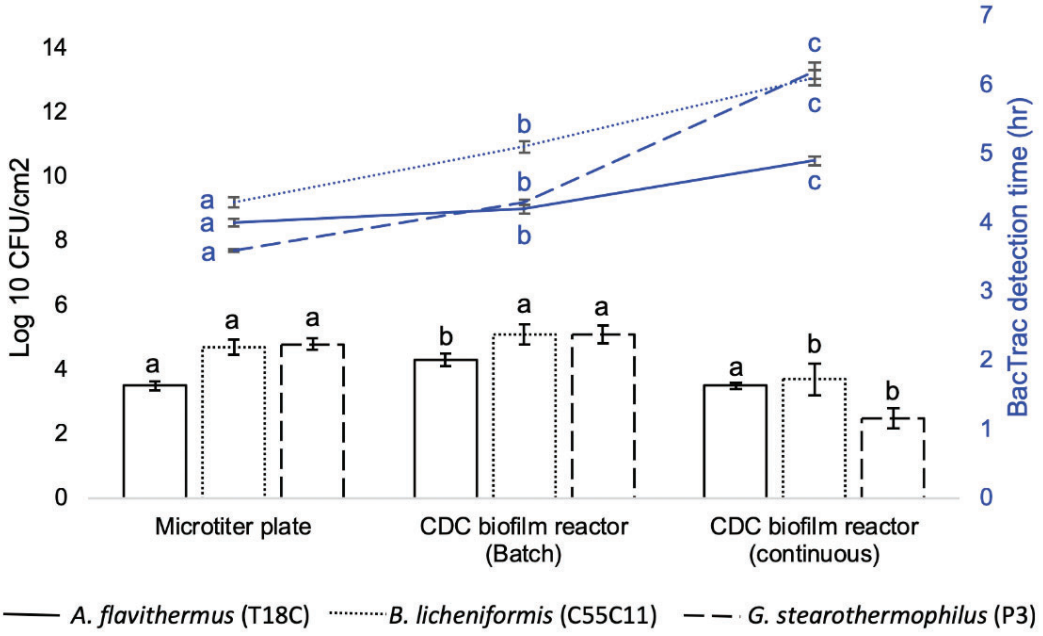


Figure 24. Comparison of biofilm formations of the thermophilic bacilli in different growth conditions. Different letters indicate statistically significant differences determined from Tukey's test and error bars are standard deviations of triplicates of each strain

Chapter 5. Discussion

This chapter addresses the cleaning efficiencies of the enzymatic treatments using proteases, endoglucanase and amylase on the removal of the biofilms. The biofilm forming capabilities of *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* in different growth environments based on various bacterial enumeration methods are also discussed.

5.1. Biofilm growth of the thermophilic bacilli

All the strains tested for *A. flavithermus, B. licheniformis* and *G. stearothermophilus* were able to form biofilms on SS coupons in a microtiter plate. The biofilm forming ability varied between strains and bacterial species, which is in agreement with many studies conducted. The plate counts for the strains of *A. flavithermus* seemed lower than the previous study with 6.2 log CFU/cm2 (Burgess et al. 2014), which could be due to the shorter incubation time (12 hours and 16 hours and a different growth media (whole milk and reconstituted skim milk)). The authors subsequently reported considerable variations present in the biofilm forming capacities of the spore-forming thermophiles such as *A. flavithermus* and *G. stearothermophilus*.

All strains of *A. flavithermus* and *G. stearothermophilus* were able to produce spores. However, spores of the strains of *B. licheniformis*, both mesophilic and thermophilic, were not detected by the standard plate count method. Li et al. (2019) established that highly heat-resistant spores which were treated at 100°C for 30 mins accounted for 31.2% of the 183 strains of *B. licheniformis* examined, while mesophilic spores treated at 80°C for 10 mins only made up 23%. Hence, the absence of the spores of the tested strains of *B. licheniformis* in the present study may imply that the strains may have been amongst the 23% of the mesophilic spores, where any potential spores could have been eliminated with the current heat treatment of 100°C for 15 minutes.

5.2. Bacterial enumeration using impedance microbiology

Using impedance microbiology (BacTrac 4300), M-values produced irregular and incompatible curves while E-values displayed suitable growth curves for all the tested strains of *A. flavithermus, B. licheniformis*, and *G. stearothermophilus*. Hence, the calibration curves were established based on E-values. The discrepancies between the two systems may be ascribed to the nature of the bacterial species as stated by Sy-Lab microbiology (2020), who corroborated that E-values have been proven for the detection of pathogens with low metabolic activities such as members of Gram-positive, heat tolerant spore formers. Flint et al. (2001) also identified that E-values were related to *G. stearothermophilus*, possibly due to dipole molecules or uncharged

substances not contributing to electrical conductivity (M-value), which may have caused changes in the electrode impedance (E-value). While most strains revealed good correlations between the plate count results derived from the calibration equations and the actual plate count, some strains were inconsistent when converted into the equivalent plate counts using the calibration equations. The slower metabolism from slow growing biofilm cells in the medium could be due to the stressed cells becoming more resistant to growth following chemical treatment and cleaning practices. These may elucidate the lack of correlations between the obtained BacTrac data and plate/spore counts of the strains as shown in Figure 7 and 8.

5.3. Enzymatic treatments on the removal of thermophilic bacilli biofilms

Amylase and protease were able to reduce bacterial cells for most strains of *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* determined from the batch biofilm reactor. The continuous flow reactor identified that amylase for *A. flavithermus*, amylase and protease for *B. licheniformis,* and endoglucanase for *G. stearothermophilus* were as equally effective as NaOH and were the most efficient cleaning regime tested. This is also substantiated by the previous finding (as acknowledged in Chapter 2.4) that proteases and amylase were the most efficient enzymes, equally effective in removing cells of *Bacillus* biofilms and *P. fluorescens* biofilms. In the same vein, the conclusion drawn by Lequette et al. (2010) also corroborated that the biofilm cells were similarly removed by NaOH and enzyme treatments. Similarly, Stiefel et al. (2016) confirmed that an active protease was able to efficiently remove *Streptococcus aureus* biofilms, whereas a combination of polysaccharides and protease was identified to be sufficient in removing biofilms of *P. aeruginosa*. Thus, it was presumed that proteases were seemingly more capable of degrading biofilms of *Bacillus* bacterial species, particularly mesophiles including *B. licheniformis*.

Ozel et al. (2017) reported that all three of mesophilic, facultative thermophilic and thermophilic bacteria tested showed the presence of cellulose-like carbohydrate in the EPSs, which get hydrolyzed and broken down by Endoglucanase. The authors additionally speculated that the examined thermophiles displayed the greatest amount of protein and extracellular DNA, accounting for thermostability and rigidity. Consequently, this conveys that proteases may possess enhanced biofilm degrading abilities than the polysaccharide degrading enzymes for thermophilic bacilli. However, the results of the different enzymatic treatments in the present research suggest that polysaccharide degrading enzymes (amylase and endoglucanase) were more efficient in reducing the biofilm cells of *A. flavithermus* and *G. stearothermophilus*. The disagreement in the finding could be attributed to the following considerations;

i. Low pH of the enzyme treatment solution - It has been established that an alkaline pH causes swelling of polymers in proteins of EPS, thereby reducing biofilm cohesiveness and promoting the removal of biofilms (Lequette et al., 2010). Conversely, an acidic pH enables biofilms to be more resistant to removal through

enhanced density and compactness of the biofilm structure. Hence, the acidic nature of the enzyme treatments may have presented an adverse effect on the removal ability on the biofilms for the enzymes.

ii. Inherent genetic characteristics of strains – Lequette et al. (2010) ascribed the discrepancies in resistance of biofilms to enzymes, cleaners and pH to genetic characteristics of individual isolates as opposed to the specific ability of a bacterial species. Consequently, this could account for the existing contradictory results when investigating the correlation between food matrix and enzymes species-specificity and removing biofilms.

iii. Different growth environments and variations between strains - Ozel et al. (2017) determined the structures of a strain of mesophile, two strains of facultative thermophile, and a strain of thermophile at different pH and temperature and in different media with NaCl. The biofilm formation and the heterogeneity of EPS compositions and biofilm matrices differ significantly between strains and species (Marchand et al., 2012). Hence, EPS and biofilm structures derived from one or two strains from each species grown in a different environment may pose difficulties in generalizing the biofilm structures of thermophilic bacilli.

iv. Complications of biofilm formation - biofilm formation is known to be a multifactorial process involving both physical and chemical attributes such as surface appendages and signalling between cells (S. G. Parkar et al., 2001). This in turn implies that biofilm development may be contingent upon various concurrent factors. Thus, considerable difficulties and inconsistencies exist in interpreting the effect of one factor on the biofilm development and enzyme treatment.

For strain D1 of *G. stearothermophilus* using the Plackett-Burman design, no enzymes were identified to significantly reduce bacterial cells, possibly owing to coagulation in the medium. As illustrated in Figure 9, conspicuous milk coagulation occurred for D1 with a measured pH of 4.2 in comparison to P3 with a pH of 5.6, which did not present notable coagulation. More prominent coagulation can be translated into enhanced biofilm forming capability as postulated by Dancer, Mah & Kang. (2009), who demonstrated that the stronger the biofilm former, the greater the coagulation developed in skim milk using strains of *E. sakazakii.* In this study, the lowered pH, as a result of lactic acid production in the microtiter plate, attracted more planktonic cells due to fouled surfaces as also substantiated by Hinton et al. (2002). Further, the degree of detachment of vegetative cells during hand rinsing in distilled water for bacterial enumeration, may have been subject to variations that affected bacterial removal. With fouling and coagulation on the surface, the fluctuations in the rinsing force may have been significant. These considerations may have contributed to the inconclusive results by the interference of the loosely attached vegetative cells on the surface, and quantification of the cell numbers is likely to have been compromised.

In comparison to *A. flavithermus* and *G. stearothermophilus*, all of the enzymatic treatments reduced the biofilm cells of both the strains of *B. licheniformis* (i.e., the plate counts were below the detection level and BacTrac detection times did not reach the set threshold in the given time). This could largely be owing to the high temperature of the enzymatic treatment,

considering the strains of *B. licheniformis* used in the study were possibly mesophilic, which were not as heat tolerant as the thermophilic strains as discussed in Section 5.1. Additionally, Ozel et al. (2017) reported that some strains of *B. licheniformis* produced EPSs consisting of trisaccharide units, exopolymer levan, and more importantly, extracellular proteins exerting anti biofilm activities. The anti-biofilm activities from the extracellular proteins may have further facilitated the removal of the bacterial cells during the enzyme treatment.

5.4. Microscopic analysis

The epifluorescence microscopy images were similar for both the CDC biofilm reactors under batch and continuous flow systems and in line with the BacTrac results, where distinguishable reductions in EPS and biofilm cells were observed following the cleaning treatments. Even so, it entails difficulties in interpreting the fluorescence images due to the staining of other organic materials such as proteins with nucleic acids as explored in the Literature review. Hence, the use of SEM allowed extensive examinations of the biofilm matrices on the coupons. The SEM images were closely linked to those of the epifluorescence microscopy images.

According to the epifluorescence microscopy and SEM images, it was evident that amylase and endoglucanase were effectively able to reduce the EPS, however, many bacterial cells or proteins still remained attached on the surfaces following treatment. On the other hand, protease was shown to perform the opposite, removing proteins more efficiently than EPS. Based on the impedance microbiology detection times, it is possible that amylase and endoglucanase were able to induce cell reductions by degrading polysaccharides/cellulose in the EPS, while protease did not sufficiently hydrolyze the extracellular proteins to access the encapsulated bacterial cells. It is also plausible that protease broke down the surface proteins as opposed to the extracellular proteins in the EPS. This contradicts the study conducted by Lequette et al. (2010), who corroborated that NaOH was not as efficient as the enzymatic treatments in removing EPS while more successfully eliminating bacterial cells on the surface. Hence, enzymes would be more suitable for in-depth cleaning of both cells and EPS of biofilms. Although the enzyme treatments failed to entirely eliminate the bacterial cells and EPS as shown in the microscopy images, the impedance microbiology results indicate that the residues on the surface were likely to be dead or inactive cells, which did not metabolize and contribute to the change in impedance. The residues remaining on the surface following the enzymatic treatments may be surface proteins or dead cells and these may be involved in providing sites for attachment of cells and formation of biofilm at a later stage (Srey et al., 2013). Therefore, with regards to the microscopy images when compared against the novel enzyme treatments, NaOH was identified to be the most promising treatment in reducing both EPS and bacterial cells, although the enzymes (amylase and endoglucanase) were also somewhat efficient in removing the EPS.

In contrast to prior research conducted on the morphology of P3 by Burgess et al. (2014) who observed a 3-dimentional structure for the strain, the SEM images of P3 of *G. stearothermophilus* in the present study showed somewhat a flatter biofilm. The discrepancies in the morphological arrangement could be due to the amount of bacterial cells attached on the surface as indicated by the difference in the plate count numbers (5 log 10 CFU/ml and 2.5 log 10 CFU/ml), along with other factors such as incubation, medium and growth conditions.

5.5. Doubling times of the thermophilic bacilli

The biofilm growth incubation time was modified from 12 hours to 24 hours for analysis using the CDC biofilm reactors (batch and continuous flow) for greater bacterial contaminations and thorough examinations on log reduction of biofilm cells following cleaning regimes.

The growth curves of T18C of *A. flavithermus*, C55C11 of *B. licheniformis*, and P3 of *G. stearothermophilus* revealed that all of the strains reached the exponential phase within the 24-hour sampling period. The doubling times and flow rates calculated from the obtained growth curves propose that *G. stearothermophilus* had the shortest doubling time and hence fastest flow rates, followed by *A. flavithermus* and *B. licheniformis*. This is in accordance with the established findings where thermophiles grow much more rapidly than mesophiles (Burgess et al., 2004). These doubling times of 49 mins, 53 mins, and 38 mins of *A. flavithermus, B. licheniformis*, and *G. stearothermophilus* were longer than the reported doubling times, 21-32 mins for the thermophiles (Burgess et al., 2009; Flint et al., 2001) and 40 - 60 mins for *B. licheniformis* (Hanlon & Hodges, 1981; Shariati, Wilfrid, Boyd, & Priest, 1995). To calculate the growth rates that best simulates the conditions in reactors, the vegetative cell growth was measured in the reactors under the identical biofilm growth environment. The longer doubling times could predominantly be as a consequence of the growth temperature fluctuations in the reactors, which may have largely affected the biofilm development. Ozel et al. (2017) also stated that while pH and salinity requirements are not directly proportional with optimal growth conditions for biofilm development, temperature was correlated with biofilm formation of both mesophiles and thermophiles. While the doubling times of *A. flavithermus* and *G. stearothermophilus* differed greatly from literature, that of *B. licheniformis* was somewhat close to the established finding, possibility due to the substantially lower growth temperature (40°C) of the bacteria and the temperature fluctuation may not have been as significant as the thermophilic bacilli grown at 55°C and 60°C.

5.6. Biofilm development in various conditions

The biofilm development under different systems was compared for strains T18C of *A. flavithermus,* C55C11 of *B. licheniformis* and P3 of *G. stearothermophilus* (Figure 24). As explained above, the biofilm incubation time was altered from 12 hours in the microtiter plate to 24 hours in the CDC biofilm reactor for greater degree of contamination and a more realistic challenge for the enzymatic treatments. There was a tendency for the BacTrac detection times to be greater for biofilms prepared in the CDC biofilm reactor under continuous flow compared with the CDC reactor running in batch mode and the microtiter plates which seemed to have produced the most biofilm. In the same vein, the plate count results suggest significantly lower bacterial cell counts for the biofilm development in the CDC biofilm reactor under continuous flow system, while those of the microtiter plate and biofilm reactor in batch mode were similar. This was not anticipated with the increased incubation time and in the presence of shear, as it was previously established that biofilm formation was enhanced under shear (Stoodley et al., 2002) and under continuous flow with the possibility of increased bacterial cell attachment and a continuous flow of nutrients (Murga et al., 2001). As addressed before, this finding could largely be attributed to the temperature fluctuations during the incubation period, as well as from the constant supply of the 4°C fresh milk into the reactor, implying that the biofilm growth temperature is likely to play a more important role than the flow condition.

Although significant differences were present between the different growth conditions tested and some discrepancies or absurd results were observed, the statistical analysis of the enzyme treatments (Section 4.4 and 4.5) suggest reproducible and congruent results; amylase for T18C (*A. flavithermus*), amylase for C55C11 (*B. licheniformis*), and P3 for *G. stearothermophilus* for biofilm cell reductions formed in the biofilm reactors under both batch and continuous flow reactor. This is due to the impact of the standardized growth conditions on the biofilm forming capabilities of the strains, hence, the assessment of the enzyme cleaning efficacy itself was not interfered.

Chapter 6. Conclusions

The present study examined the efficacy of novel enzymes (amylase, proteases and endoglucanase) on the removal of thermophilic bacilli biofilms on dairy manufacturing surfaces. Following the initial strain selection stage for the determination of strong biofilm formers, two strains proceeded for further analysis using enzymes.

Based on the Plackett-Burman experimental design, amylase and protease (CB14057) for *A. flavithermus*, protease (CB14057) and endoglucanase (CB13961) for *G. stearothermophilus* had produced a reduction in biofilm. Enzyme removal of *B. licheniformis* biofilms was inconclusive, with all of the enzymatic treatments appearing to be efficient. Considering the results of the Plackett-Burman experiment of *B. licheniformis*, amylase and protease (CB14057), which were shown to reduce the biofilm cells of the other thermophiles tested, were examined for *B. licheniformis* strains. investigations conducted on biofilms developed in a CDC biofilm reactor under the batch system suggest significant efficacy of amylase, protease and the combination of amylase and protease for *A. flavithermus* and *B. licheniformis*, and amylase for *G. stearothermophilus*. The subsequent experiment carried out in the reactor under the continuous flow system confirmed the similar results obtained from the batch examination. As demonstrated, amylase for *A. flavithermus*, amylase, protease and the combination of amylase and protease for *B. licheniformis* and endoglucanase for *G. stearothermophilus* had a significant impact on reducing biofilm cells and EPS. This disagrees with the previous studies, where proteases were the most efficient enzyme on thermophiles, and the discrepancies in the findings could be attributed to a number of factors such as i) low pH of the enzyme treatment solution, ii) inherently varied genetic characteristics of strains, iii) different biofilm growth environments, and iv) multifactorial process of biofilm development.

Chapter 7. Recommendations and future research

The conclusion and discussion have presented the following recommendations for future research directions and questions;

I. Conduct investigations on enzymatic treatments in a flow system to simulate the cleaning regime in the industry, as cleaning takes place in high flow areas for hard-to-clean surfaces.
II. Carry out scale-up pilot plant or full factory trials. Enzymatic treatments on laboratory-grown biofilms may implicate different applications than the industrial situations, as it has been established that spores produced by thermophilic bacilli in a milk powder factory are more heat-resistant than those produced in laboratory media.
III. Research into reusing the enzyme solutions. One of the considerable obstacles encountered when implementing the enzymatic cleaners is the high cost related to the enzyme cleaning practices, hence investigating opportunities to minimise the cost impact is indispensable. Reusing the enzyme solution may present plausible and promising outcomes for the method to become more adaptable in the industry.
IV. Examine the enzymatic treatments at a lower temperature for *B. licheniformis* to ascertain whether the significant reduction on the bacterial cell counts were associated with the high temperature or the break-down of the EPS and biofilm matrices from the enzymes.
V. Conduct in-depth analysis on the composition of the EPS and biofilm matrices of the strains of *A. flavithermus, B. licheniformis* and *G. stearothermophilus* to acquire comprehensive knowledge on the interactions between the enzymes and biofilms.
VI. Test the enzyme cleaners on multispecies biofilms, as biofilms formed in the industry may not necessarily be mono-species biofilms.

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