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Novel hyperthermoacidic archaeal enzymes for removal of thermophilic biofilms from stainless steel

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Abstract

Aims: To test the efficacy of novel hot/acid hyperthermoacidic enzyme treatments on the removal of thermophilic spore-forming biofilms from stainless steel surfaces.

Methods and results: The present study measured the efficacy of hyperthermoacidic enzymes (protease, amylase, and endoglucanase) that are optimally active at low pH (≈3.0) and high temperatures (≈80°C) at removing thermophilic bacilli biofilms from stainless steel (SS) surfaces. Plate counts, spore counts, impedance microbiology, as well as epifluorescence microscopy, and scanning electron microscopy (SEM) were used to evaluate the cleaning and sanitation of biofilms grown in a continuous flow biofilm reactor. Previously unavailable hyperthermoacidic amylase, protease, and the combination of amylase and protease were tested on *Anoxybacillus flavithermus* and *Bacillus licheniformis*, and endoglucanase was tested on *Geobacillus stearothermophilus*. In all cases, the heated acidic enzymatic treatments significantly reduced biofilm cells and their sheltering extracellular polymeric substances (EPS).

Conclusions: Hyperthermoacidic enzymes and the associated heated acid conditions are effective at removing biofilms of thermophilic bacteria from SS surfaces that contaminate dairy plants.

Significance and impact of study

Hyperthermoacidic archaeal enzymes (HTA-enzymes) function optimally in conditions that are toxic to most microbes and are tested here for cleaning and removal of biofilms. *Anoxybacillus flavithermus*, *B. licheniformis*, and *G. stearothermophilus* are abundant thermophilic biofilm-formers in the dairy industry. Due to the inadequate hygienic performance of conventional cleaning approaches on biofilms, enzymes have been studied as an alternative, predominately on non-spore-forming mesophilic or psychrophilic biofilms at a basic or neutral pH at ≈60°C. This study showed the potential for HTA-enzymes as natural and effective cleaning and sanitation products for the removal of biofilms. Additionally, the use of enzymatic cleaning formulations will reduce the environmental impacts caused by the disposal of traditional cleaners such as NaOH and quaternary ammonium compounds (QACs).

Keywords: archaea, spores, *Anoxybacillus flavithermus*, *Bacillus licheniforms*, *Geobacillus stearothermophilus*, cleaning, protease, amylase, endoglucanase, dairy, HTA-enzymes

Introduction

As environmental concerns and regulatory restrictions begin to limit the use of synthetic chemicals for the essential cleaning and sanitation functions in industry, alternative cleaning and sanitation approaches become more valuable. The control of biofilms in the food industry, in particular dairy manufacturing plants, using traditional methods is a challenge (Knight 2015). The promise of enzymatic cleaning for food processors, and many other industries, has been reported extensively in the scientific literature (Liu et al. 2014, Nahar et al. 2018). Broadly speaking, hydrolytic enzymes are biocatalysts that specifically degrade a class of biomolecules (Alderson et al. 2012). Importantly, both biofilms and foods are primarily biomolecules that have cognate enzymes that can degrade them. Biofilms are agglomerations of microbial cells adhered to surfaces embedded in a self-produced matrix of extracellular polymeric substances (EPS). The composition of EPS molecules and covalent linkages varies widely between microbial species and even within species depending on several factors (Flemming 2016), making biofilm suppression, removal, and sanitation significant challenges in food processing, medical, surgical, and other biomolecule-intensive industries. Once developed, biofilms are highly resistant to antimicrobial compounds and cleaning or disinfection procedures, posing a significant safety and quality concern for food and other industries (Austin and Bergeron 1995). Of significance, the components of biofilm EPS are primarily polysaccharides, proteins, and nucleic acids (Austin and Bergeron 1995, Marchand et al. 2012, Flemming 2016). All these molecule classes have evolved to depolymerize (degrade) these EPS biomolecules. These ideas have given rise to a growing interest and investigation of enzymatic approaches to address the persistent and important issues of biofilm removal in industrial processes.

Mesophilic organisms grow near average ambient temperatures and concomitantly have enzymes that operate optimally in those same temperature ranges (∼20–40°C). In contrast, thermophiles have optimal growth temperatures well above this range (50–70°C) (Burgess et al. 2014). Hyperthermophilic organisms from the divergent archaeal domain of life, have optimal growth and enzyme functions in the approximate

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Table 1. Basic physical and biochemical characteristics of HTA-enzymes investigated in this study (CinderBio).

Catalogue	Class	Optimal pH	pH range	Optimal temperature	Temp range	Half-life (at optima)
CB-13961	Endoglucanase	2.0	1.2–4.5	75°C	60–85°C	To be determined
CB-13184	α-Amylase	3.5	2.2–4.5	100°C	80–110°C	>288 h
CB-14057	Endoprotease	3	1.5–4.0	70°C	38–100°C	240 h

range of 70–100°C (Woese et al. 1990). In addition to hyperthermophiles, some organisms have evolved not only to extremely hot environments, but also to highly acidic conditions (pH ~3). This group of archaeal organisms is known as hyperthermoacidophiles and has evolved enzymes that function optimally in acidic and extreme heat environments (Table 1). Hyperthermoacidic enzymes have unique practical properties with respect to shelf life, half-life, and fast reaction times, among other valuable characteristics. In addition to ultra-stability, hyperthermoacidic archaeal enzymes (HTA-enzymes) function optimally in conditions that are toxic to nearly all organisms. Here we undertake, for the first time, studies to investigate the utility of HTA-enzymes and the antimicrobial conditions in which they operate for the practical cleaning and sanitation of industry-relevant biofilms.

Major drawbacks of enzymatic cleaners have historically included the limited thermal and pH operational ranges of enzymes, short shelf lives, slow reaction rates, and limited formulation options. HTA-enzymes (≈80–100°C and pH 2–4) have become available as potential sources of highly active and stable industrial enzymes (Yannone and Barnebey 2012, Yannone et al. 2016). These enzymes not only function optimally in conditions that are toxic to most bacterial species, but the extreme temperature and pH are also capable of chemically hydrolyzing (breaking down) nucleic acids, an important part of many biofilm EPS structures (Flemming 2016). For the first time, we investigate enzymatic cleaning and sanitation under heated acid conditions previously incompatible with functional enzymes. Here, we test a multifaceted approach with heated acid serving to kill microbes and hydrolyze nucleic acids in biofilm EPS while simultaneously degrading biofilm polysaccharides and protein component molecules with targeted enzymatic activities.

This study investigates biofilm sanitation and EPS removal for three commercially relevant thermophilic organisms isolated from dairy processing facilities. Endospore-forming thermophilic bacilli such as *Anoxybacillus flavithermus, Bacillus licheniformis*, and *Geobacillus stearothermophilus* are abundant microorganisms present in dairy products, acting as hygienic indicators due to their thermostability and the ability to form spores and grow over a wide temperature range (Burgess et al. 2010). These thermophiles facilitate biofilm development and sporulation, resulting in spoilage and rancid flavors through acid and enzyme production, and are resistant to removal with standard Clean In Place (CIP) approaches. This study examines the efficacy of HTA-enzyme treatments on established thermophilic biofilms of species isolated from dairy operations.

Materials and methods

Bacterial strains and culture conditions

Strains of *A. flavithermus* (T18C), *B. licheniformis* (C55C11), and *G. stearothermophilus* (P3) used in the study are dairy isolates from a milk powder manufacturing plant, whey protein, and evaporated milk, respectively. These isolates were identified using specific DNA PCR primers (Flint et al. 2001). Cultures were maintained at -80°C in Cryobank vials (Mast Group Ltd., Liverpool, UK). For use, one bead was transferred and grown overnight on Milk Plate Count Agar (MPCA, OXOID, Hampshire, England) using the 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 al. 2019). The broth was incubated for 8 h at 55°C, 40°C, and 60°C for *A. flavithermus, B. licheniformis*, and *G. stearothermophilus,* respectively, and mixed by vortex to allow the 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, the optical density (OD) of the growth was measured at a 600 nm wavelength using Spectrostar Nano (BMG Labtech, Ortenberg, Germany) and adjusted to ~0.5 OD to achieve a cell density of 5–6 log CFU/mL to be used as a starting culture.

Biofilm development

For biofilm development under shear in a dynamic state, a Centers for Disease Control (CDC) biofilm reactor (Biosurface Technologies Corporation, MT, USA) was used (Supplementary Fig. S1) (Goeres et al. 2005). The outlet of the reactor and air tubing on the top were thoroughly blocked using a 0.45 µm filter and stopper to prevent any bacterial contamination via air. For coupon preparation, SS coupons (304 grade with a 2B surface finish) with radius = 12.7 mm, thickness = 3.8 mm, and total area = 4.05 cm^2 were washed, with gentle agitation, in 1% NaOH (Merck, Damstadt, Germany) for 10 min, followed by rinsing with distilled water. The coupons were then immersed in acetone (Univar, IL, USA) for 10 min to remove any grease, rinsed by distilled water, and autoclaved. The reactor with SS coupons fitted into the cylindrical Teflon holder was autoclaved prior to use.

Supplementary Fig. S1 is a schematic diagram of the continuous-flow CDC biofilm reactor. The ultra-high-temperature (UHT) whole milk was prepared from whole milk powder (Fonterra, Palmerston North, New Zealand) at the FoodPilot, Massey University. This milk was assumed to be sterile due to the UHT process (Tetra Pak 2023). The milk powder was reconstituted in cold water using a FP004 Cowles mixer (Massey University, Palmerston North, New Zealand) to produce 11% milk powder at a mixer speed of 35 rpm and was hydrated for 20 min. The milk was then homogenized (Rannie, Copenhagen, Denmark) at 100 and 50 pa and processed through UHT (Massey University, Palmerston North, New Zealand). The UHT milk was collected into autoclaved 20 L plastic cans, in a laminar cabinet. A total of 300 mL of 8 h cultures of the three thermophiles grown in TSB were inoculated into each can containing 20 L of the UHT milk (1.5% bacteria culture in the solution). The can of UHT milk was stored in the cold room, and the milk was pumped (MasterFlex, IL, USA) at the calculated flow rates through a rubber tube to the reactor on a hot plate set to the appropriate temperature and an agitation of 100 rpm for 24 h. Following incubation, the Teflon holders were separated from the reactor, and the SS coupons were removed and washed in 40 mL of sterilized distilled water three times prior to microbial enumeration and analysis. The analysis was conducted in triplicate.

Flow rate calculations

Doubling times of the three thermophiles were measured from growth curves, and these were used to determine the flow rate. Eight-hour 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 containing 400 mL of UHT milk, without SS coupons fitted. The reactors were then placed on hot plates heated to the specified temperatures in a laminar flow cabinet. Starting from time zero, 1 mL of samples were taken every 2 h up to 12 h from each of the three reactors aseptically, followed by 10-fold serial dilution and droplet plating in triplicate on MPCA. The plates were incubated for 24 h for plate counting, 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, the doubling time was calculated as

$$lln\left(\frac{C_f}{C_i}\right) = k\left(t_f - t_i\right) = \frac{lln\,2}{k}, \tag{1}$$

where k is the 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 at C_i. Flow rates were established such that the total volume of the milk in the reactor (400 mL) was replaced with fresh milk in the time taken for the numbers of bacteria to double.

Biofilm treatments

Preliminary tests for biofilm removal of each strain were first tested in microtitre plates using each enzyme individually and combinations of each enzyme. The most successful enzyme treatments were chosen to test on stainless steel (SS) coupons, and these are presented in this paper.

After rinsing the coupons containing biofilm, the coupons were treated with enzyme solutions. For enzymatic solution preparations, industrial-grade preparations of hyperthermoacidic amylase (CB13184), protease (CB14057), and endoglucanase (CB13961) (CinderBio, CA, USA) were diluted 100 times in a phosphate citric acid buffer pH = 3.0. For enzymatic formulations representing multiple enzymes, equal amounts of each enzyme were added to the buffer to achieve 1:100 dilutions (i.e. for a solution containing two different enzymes, 0.5 mL of each enzyme was added into 100 mL of the phosphate–citric acid buffer). To make the phosphate citric acid buffer, 2.84 g of sodium phosphate dibasic (Na_3PO_4) (Univar, IL, USA) and 7.69 g of citric acid (Univar, IL, USA) were dissolved in 1 L of distilled water, after which the pH was adjusted to ∼3.0 and autoclaved. The enzyme solutions were stored in a water bath at 85°C for future use. Biofilm coupons were immersed and treated in 25-mL vials containing 10 mL of the enzyme solutions for 20 min at 85°C in a water bath. In addition to the enzyme treatments, examinations on biofilm coupons before cleaning (BC) without any treatment, control (C) with 85°C sterile water, and with treatment with 1% NaOH were carried out in conjunction with the enzymatic cleaning to establish a comparison. SS coupons were rinsed in sterile distilled water after treatment before analysis. All examinations were conducted in triplicate. HTA-enzyme treatments were assayed to remove biofilm of a thermophilic biofilm-forming strain of *B. licheniformis* C55C11, which was isolated from a dairy processing facility. In this experimental set, HTA-amylase, HTA-protease, and both enzymes were used in combination.

Plate count and spore count of biofilm cells

SS coupons were placed in 25-mL vials containing 10 mL of 0.1% peptone water (PW) (Merck, Damstadt, Germany) and 15 g of 3.7–4.1 mm 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 9 mL 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 h. For examining spores present in the samples after plating the viable biofilm cells, the 25-mL vials containing the SS coupons were placed in boiling water for 15 min to quantify spores that were able to survive high temperatures. After heating, the vials were cooled 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 h. This is based on one of the many standard methods for counting thermophilic bacteria (Frank et al. 2002). For spore counts, biofilms were detached from the coupons and heated to 100°C for 15 min before plating. We also imaged cells and EPS remaining on the coupons with both epifluorescent and scanning electron microscopy (SEM), respectively.

Impedance microbiology

The BacTrac 4300^{TM} (SyLab, Purkersdorf-Vienna, Austria) is a sensitive and efficient method to measure viable cells and spores (Flint and Brooks 2001). Sensitive, in that even one cell on a sample surface can be detected. Efficient in that damaged cells and spores are given the best opportunity to grow and be detected in a broth medium, and less time is needed to prepare samples for analysis. The BacTrac measures the changes in impedance in the medium derived from microbial metabolism and resistance because of bacterial growth. Figure 1 depicts the schematic diagram of the BacTrac calibration for measuring biofilm cells. Eight-hour 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 incubated in BacTrac vials containing 10 mL of TSB in triplicate. The plates were incubated for 24 h at 55°C, and the BacTrac vials were incubated at the appropriate temperature (the identical growing temperatures in TSB) for 24 h for the change in impedance. The plate count in $\log_{10}$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 equation was later used to convert the BacTrac threshold times into the equivalent $\log_{10}$CFU/mL for

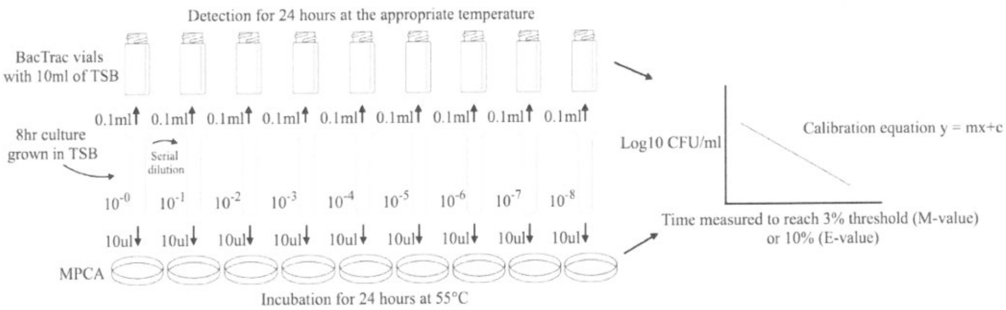

Figure 1. Schematic diagram of developing a calibration curve using BacTrac 4300.

quantifying biofilm cells. For analysis using impedance microbiology, rinsed SS coupons were each placed in BacTrac vials containing 10 mL TSB and incubated for 24 h at the appropriate temperatures for the detection of impedance change. All experiments were carried out in triplicate.

To assess the relative effects of the various cleaning and sanitation treatments on established thermophilic biofilms and their spores, we made four measurements for each strain before and after treatment. For cell and spore viability, we quantified viability with total plate counts (TPCs) and impedance growth measurements (BacTrac).

Epifluorescence microscopy

Epifluorescence microscopy was used to provide a visual interpretation of both the microbial cell content and organic material. The method followed the basic procedure outlined by Whitehead et al. (2010). Fluorochrome acridine orange (Sigma Aldrich, MO, USA) of weight 10 g was dissolved in 1 L of 0.1 M phosphate buffer saline (PBS) and filtered through a 0.2 µL Sartorius filter. To make PBS, 8 g of NaCl, 200 mg of KCl, 1.44 g of Na_2HPO_4, and 240 mg of KH_2PO_4 (Univar, IL, USA) were dissolved in 800 mL 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 immersed in the acridine orange solution for 2 min. The coupons were gently rinsed with distilled water three times and air dried. Each coupon was mounted on a glass slide and examined under an Olympus microscope B × 53 with a FITC light excitation filter block using CellsSens Dimension software.

Scanning electron microscope

Scanning electron microscopy (SEM) was used to provide a more detailed interpretation of the microbial and organic material on the surfaces. This followed the basic method outlined by Whitehead et al. (2010). The coupon samples were placed in primary fixative (modified Karnovsky's fixative, 3% gluteraldehyde, 2% formaldehyde in 0.1 M sodium cacodylate, pH 7.2), and the samples were fixed for at least 8 h at room temperature. Following fixation, samples were washed three times (10–15 min each) in phosphate buffer (0.1 M, pH 7.2) followed by dehydration in graded ethanol series [25%, 50%, 75%, 95%, 100% (v/v)] (Ajax Finechem, Thermofisher Scientific) for 15 min each and a final 100% ethanol wash for 1 h. 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, sputter coated with $\sim$100 nm of gold (Baltec SCD 050 sputter coater), and analyzed in the FEI Quanta 200 Environmental Scanning Electron Microscope at an accelerating voltage of 20 kV.

Statistical analysis and software

All data points were reported as means with standard deviations. Data acquired were analyzed with one-way analysis of variance (ANOVA, $P < 0.05$) on SPSS 18.0 and graphed using Origin 8.5 (MA, USA).

Results and discussion

Flow rate and doubling time determination

To determine the doubling times and flow rates of the strains required for experimenting in 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 (Supplementary Fig. S2). With the starting bacterial concentrations ranging from 4 to 5 $\log_{10}$CFU/mL, the exponential phase was reached within 24 h. *Bacillus licheniformis,* both mesophilic and thermophilic, obtained a gradual incline, while the two thermophilic species displayed steeper exponential growth. Based on the exponential phase from the growth curves acquired, doubling times and flow rates were calculated as shown in Supplementary Table S2. As previously revealed in Supplementary Fig. S2, the results were as anticipated, with *B. licheniformis* having the longest doubling time (53 min), followed by *A. flavithermus* (49 min) and *G. stearothermophilus* (38 min). 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, using Equation (1).

Impedance microbiology detection system and calibration curves

Based on the results of impedance detection for both *M*-values (3% threshold) and *E*-values (10% threshold), *E*-value growth curves were reproducible and applicable, while curves derived from *M*-values displayed inconsistent and contradictory growth curves (data not shown). 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

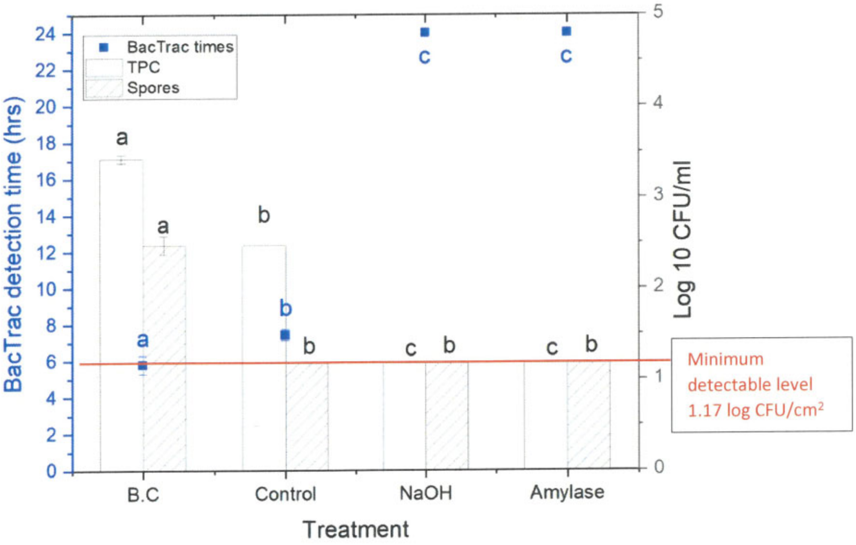

Figure 2. BacTrac hours, TPC, and spore results of strain T18C of *A. flavithermus* grown in a CDC biofilm reactor in a continuous flow system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test, and error bars are standard deviations of triplicates of each strain.

correlations with $R^2 > 0.9$ (Supplementary Table S1). 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 that may have been due to the severe treatment they were exposed to) for the strains tested. Hence, the BacTrac detection results for the present study were expressed and analyzed in hours as opposed to their log counts.

For the *A. flavithermus* T18C biofilms, the TPC and spore counts following NaOH and HTA-amylase treatments at 85°C for 20 min were both below the detection thresholds and markedly reduced from pre-treatment levels (Fig. 2). Impedance measurements of viability corroborated the low to no viability results after these two treatments. Inspection of the SEM images for NaOH treated biofilms (Figs 3 and 5) shows substances remaining on the surface in peculiar nodular forms, presumably protein or EPS residues in forms that are structurally altered by NaOH treatment but not detached from the surface. In contrast, no nodules are observed following HTA-amylase treatment and EPS is notably reduced on the coupon surface. Clusters of acridine orange-stained material are plainly visible on the surface after HTA-amylase treatments but not the NaOH treatment and with the heat-only control (Fig. 3). Both NaOH and amylase treatments reduced microbial metabolism below detection thresholds after measurements with three separate measurements while the heat-only control showed robust outgrowth with impedance measurements (Fig. 2). These data indicate that the visualized acridine orange staining on the HTA-amylase treated coupons does not reflect viable cells or spores. Regardless, inspection of the SEM image after HTA-amylase treatment reveals the heat/acid/HTA-amylase treatment effectively reduced *A. flavithermus* cell and spore viability and reduced the EPS remaining bound to the SS coupon in these experiments.

The *B. licheniformis* isolate showed very robust biofilm development based on cell plate counts, spore counts, impedance measurements, and SEM imaging (Figs 4 and 6). Despite very robust biofilm development, the plate and spore counts following NaOH and HTA-enzyme treatments were below the detection limits of these methods (Fig. 6). Again, in contrast to the NaOH and enzyme treatments, the heat-only control showed significant outgrowth with the impedance measurements. Like the *A. flavithermus* microscopy images (Fig. 3), the images of *B. licheniformis* biofilms after treatments show acridine orange-staining material remaining on enzyme-treated coupons but no viable cells or outgrowth. Inspection of the SEM images shows the individual HTA-enzyme treatments both have significant residue remaining on the coupons, with the amylase/protease combination having the least residual EPS (Fig. 4). Taken together, these data indicate that HTA-enzyme treatments are effective at removing robust *B. licheniformis* biofilms attached to SS, and combinations of HTA-enzymes may be more effective at removing EPS.

Lastly, we tested the performance of HTA-enzymes for removing *G. stearothermophilus* biofilms. In these experiments, we tested HTA-protease, HTA-endoglucanase, and both enzymes in combination. The plate counts for *G. stearothermophilus* biofilms after all treatments were all below the detection limits (Fig. 7). However, impedance detection times show no outgrowth within 24 h for NaOH, but viable cell outgrowth being detected in all enzymatic treatments. The SEM images for strain *G. stearothermophilus* (Fig. 5) show only minor amounts of residue left on the surfaces following all treatments; however, treatments with NaOH and endoglucanase seemed to produce the cleanest surfaces. Notably, *G. stearothermophilus* biofilms in these images are nearly completely removed even with heat-only controls, despite significant outgrowth in the control.

Here, we investigate for the first time the use of ultrastable HTA-enzymes and the associated heated acid conditions to clean and sanitize industrially relevant biofilms of thermophilic bacteria. The hyperthermoacidic protease, en-

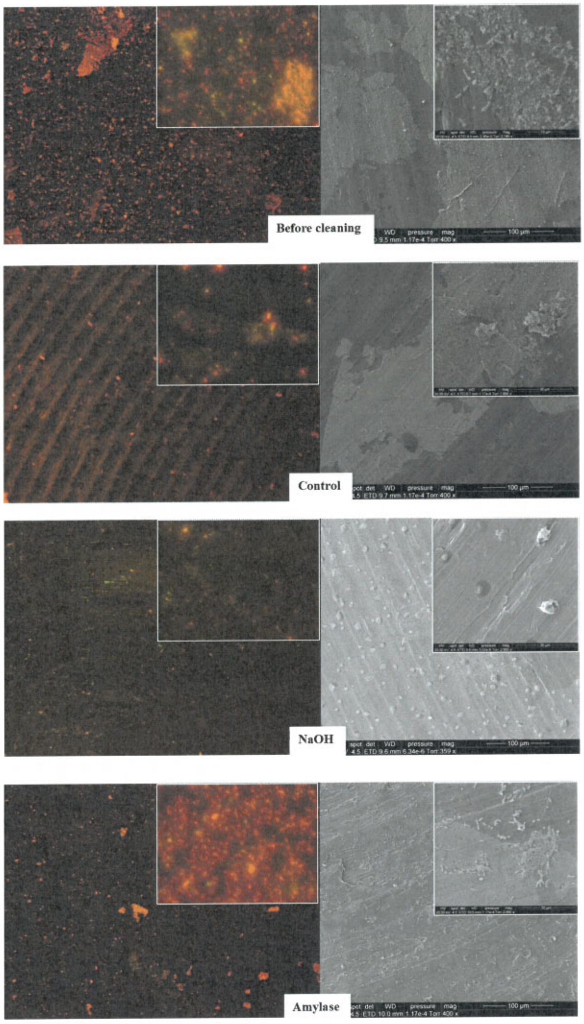

Figure 3. Images of the biofilm of T18C of *A. flavithermus* grown in a CDC biofilm reactor in a continuous flow system under epifluorescence microscopy using acriding orange (left). The big image is 10 mm and the small image is 4 mm in scale. Images on the right are SEM images with scales shown.

doglucanase, and amylase treatments all show significant sanitation and removal of biofilm EPS from the SS coupons. Unsurprisingly, the performance of NaOH and the different HTA-enzyme treatments varied in EPS removal between the various species, likely due to variations in the biofilm makeup between species and the cognate enzymatic activities. These observations are consistent with biofilm-to-biofilm variations in enzyme performance observed in many studies (Nahar et al. 2018). For example, in one case, serine proteases performed better for the removal of *Bacillus* sp. biofilms, while an amylase performed better with *P. fluorescens* biofilms (Lequette et al. 2010). Similarly, an active protease alone was able to efficiently remove *Staphylococcus aureus* biofilms, whereas a combination of polysaccharidase and protease was required to effectively remove biofilms of *P. aeruginosa* (Stiefel et al. 2016). The authors ascribed these discrepancies to the significant variations in the heterogeneities of biofilms developed from each strain, and subsequently proposed that combinations of enzymes would be beneficial for removing biofilms from surfaces (Meireles et al.

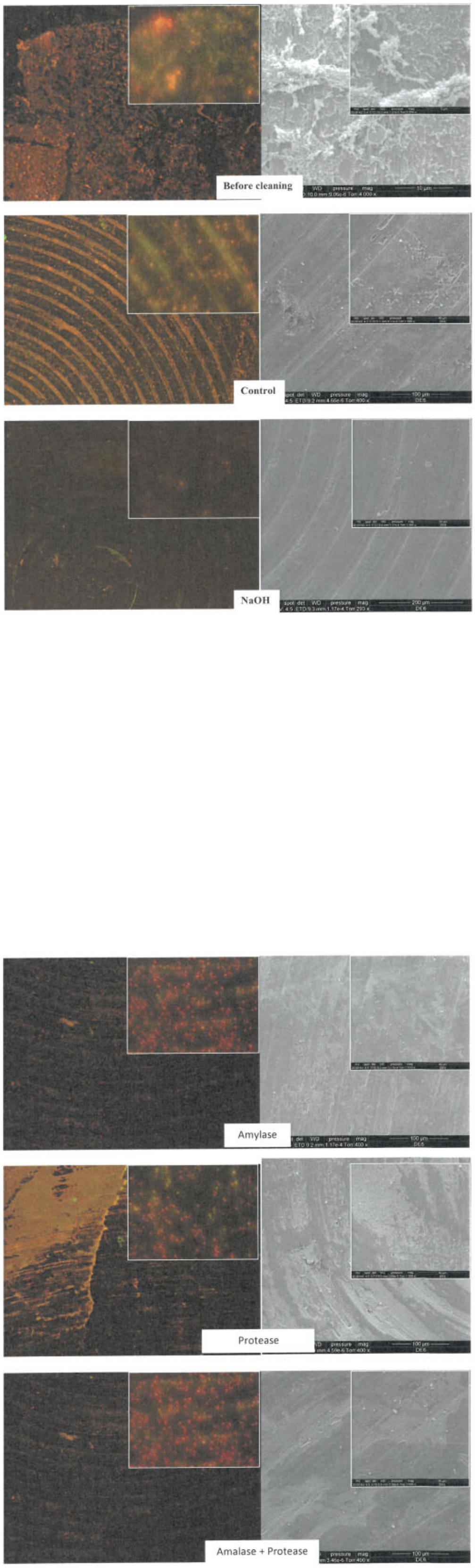

Figure 4. Images of the biofilms of C55C11 of *B. licheniformis* grown in a CDC biofilm reactor in a continuous flow system under epifluorescence microscopy using acridine orange (left). The big image is 10 μm and the small image is 4 μm in scale. Images on the right are SEM images with scales shown.

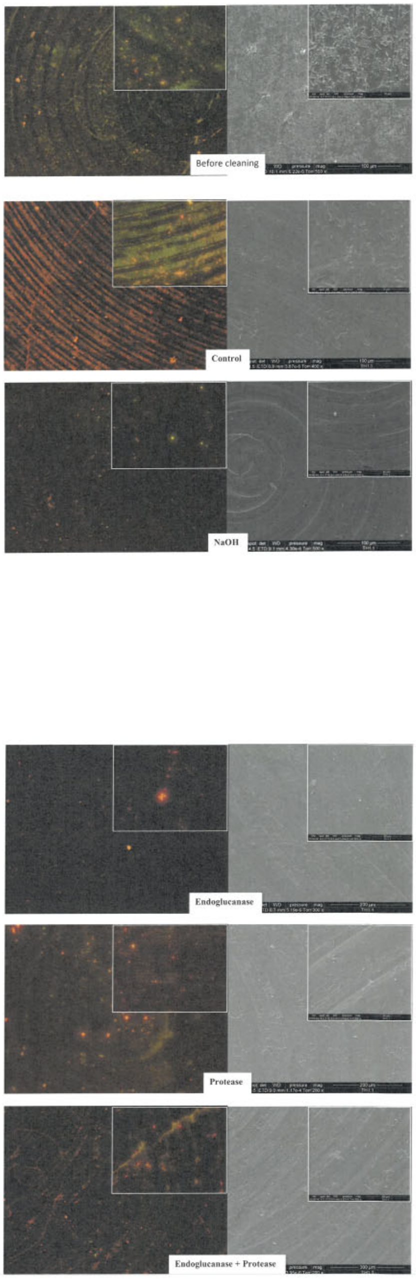

Figure 5. Images of the biofilms of P3 of *G. stearothermophilus* grown in a CDC biofilm reactor in a continuous flow system under epifluorescence microscopy using acridine orange (left). The big image is 10 μm and the small image is 4 μm in scale. Images on the right are SEM images with scales shown.

2016). Importantly, co-formulation of sets of enzymes with a protease presents inherent challenges, as the protease degrades proteins, and all enzymes are proteins. Notably, a novel characteristic of HTA-enzymes is that most HTA-enzymes are highly resistant to proteolytic digestion and therefore may permit stable multi-enzyme formulations, including proteases for a broader-spectrum enzymatic biofilm cleaning and sanitation formulation (Yannone et al. 2021). A second characteristic of HTA-enzymes that is relevant for cleaning and sanitation is the remarkable half-lives of these newly available enzymes (see Table 1). The determined half-lives are typically expressed in days, and not hours, despite the harsh 80°C and pH 3 conditions. This ultra-stability could facilitate longer contact times in cleaning applications and enzyme re-use in CIP and/or soak-tank contexts. Finally, the heated acid conditions in which HTA-enzymes function optimally have inherent antimicrobial activity. Taken together, our results reveal the potential for exploiting the ultra-stable heat and acid-loving nature of HTA-enzymes as part of effective cleaning regimens for biofilm remediation in food processing facilities.

As mentioned previously, biofilms differ widely between species as well as within a species depending on culture conditions. Previously, 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 is hydrolyzed and broken down by endoglucanase enzymes. The authors additionally speculated that the examined thermophiles displayed the greatest amount of protein and extracellular DNA, accounting for thermostability and rigidity. Consequently, this suggests that proteases may possess enhanced biofilm-degrading abilities relative to the polysaccharide-degrading enzymes for thermophilic bacilli. However, the results of the different HTA-enzymatic treatments on the thermophilic bacilli biofilms in the present research indicate that polysaccharide-degrading enzymes (amylase and endoglucanase) were more efficient in reducing the EPS of *A. flavithermus* and *G. stearothermophilus*. Here, protease alone was the least efficient cleaning treatment for *G. stearothermophilus*, as shown by the short BacTrac detection times. Importantly, unlike chemical cleaners, enzymes have remarkable specificity, and in the case of proteases, they often queue their activity on two or three specific amino acids as they appear in a protein sequence. Protease specificity, together with the varied protein sequences that predominate any given biofilm, can lead to disparate observations depending not only on the microbial species, but also on the protease and its inherent cleavage specificity on proteins within any biofilm. In other words, it may not be reasonable to expect extremely divergent proteases to be equally effective on biofilms, even those from the same species.

According to the epifluorescence microscopy and SEM images, it was evident that the amylase and endoglucanase, in combination with the hot/acidic reaction conditions, were effectively able to reduce the EPS. However, significant acridine orange-staining material remained attached on the surfaces following treatments, despite no or disproportionate outgrowth or plate counts from those samples. Therefore, it is difficult to confidently interpret the epifluorescence with respect to viable cell counts. Notably, acridine orange staining of non-cell organic materials such as proteins and DNA has been reported, as well as the variability and a lack of accuracy of acridine orange in the differentiation of live and dead cells (Hood and Zottola 1995). This finding is in accordance with Blackman and Frank (1996), who speculated that biofilm quantification using epifluorescence microscopy is often correlated with the underestimation of biofilm cells from not accounting for the thickness, and the overestimation of cells due to the staining of some EPS. Hence, the use of SEM allowed direct examination of the biofilm matrices on the coupons and is likely the more reliable measurement presented here.

It is not surprising that variations in the molecular constituents and the specific covalent bonds in molecules con-

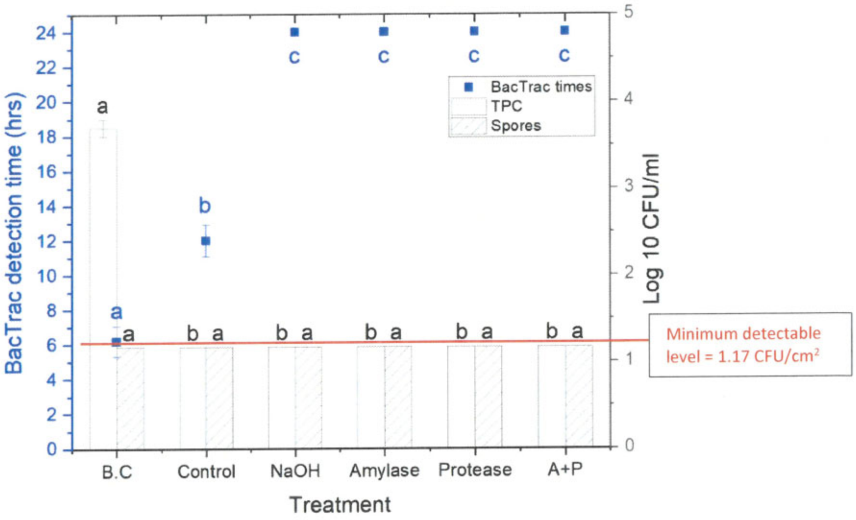

Figure 6. BacTrac hours, TPC, and spore results of strain C55C11 of *B. licheniformis* grown in a CDC biofilm reactor in a continuous flow system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test, and error bars are standard deviations of triplicates of each strain.

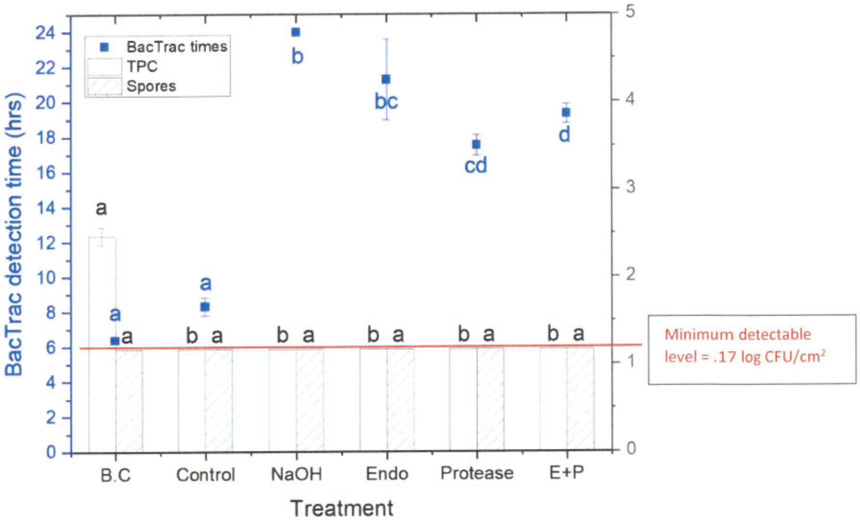

Figure 7. BacTrac hours, TPC, and spore results of strain P3 of *G. stearothermophilus* grown in a CDC biofilm reactor in a continuous flow system. Results are means and standard deviations from three replicates. Different letters indicate statistically significant differences determined from Tukey's test, and error bars are standard deviations of triplicates of each strain.

stituting EPS would impact the efficacy of enzymes, which have evolved highly specific chemical recognition and activities. The HTA-amylase was more effective for *A. flavithermus*, while the HTA-amylase, HTA-protease, and a combination of both were more effective for removing *B. licheniformis*, and the HTA-endoglucanase performed best in reducing biofilm cells and EPS with *G. stearothermophilus*. The cleaning results were as good as those obtained with standard caustic cleaning and demonstrate an alternative, environmentally friendly approach to cleaning dairy plant surfaces. Multiple HTA-enzyme formulations and pilot-scale testing under conditions that simulate the dairy industry are underway to provide confidence for industrial applications and to develop a broad-spectrum enzymatic cleaner for biofilms.

This present trial examined the removal of biofilms of thermophilic spore-forming bacteria common in dairy manufacturing. Spores are important and the most resistant forms of these bacteria (Seale et al. 2015). This study did not specifically examine the efficacy of these enzymes on removing spores from surfaces and is something to study in future studies.

The hyperthermoacidic protease, endoglucanase, and amylase treatments in these preliminary trials, all show potential for the removal of biofilm EPS from thermophilic *Bacillus* biofilms from SS. These enzymes can be considered as effective, environmentally friendly cleaners for use in dairy manufacture. The benefit of using enzymes to treat biofilm is that enzymes target the predominant component (e.g. EPS) on contaminated surfaces, and this may, in some cases, be more effective than a general chemical cleaner. While the acid pH used for these enzymes will need neutralizing before disposal, there is potential to reuse these enzymes for multiple cleans, reducing the amount of effluent from a manufacturing plant. This will be investigated in future studies.

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest statement: Three of the authors (A.B., H.K.K., and S.M.Y.) work for the company that produces the enzymes used in this study. Enzymes used in this study can be purchased at CinderBio.com.

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Author contributions

Yurina Nam (Data curation, Formal analysis, Investigation, Writing – original draft), Adam Barnebey (Investigation, Resources, Writing – review & editing), Hayoung K. Kim (Investigation, Resources, Writing – review & editing), Steven M. Yannone (Conceptualization, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing) and Steve Flint (Conceptualization, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing).

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