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Sequential treatment of psychrotrophic pseudomonad biofilms with sodium hydroxide and commercial enzyme cleaners

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ABSTRACT

Pseudomonads are psychrotrophic spoilage bacteria that can form biofilms at the air-liquid interface. Food processing utensils and equipment often facilitate the air-liquid interface biofilm formation. Pseudomonads produce thermostable enzymes and pigments that affect the organoleptic quality of perishable food products. In this study, *Pseudomonas lundensis*, *Pseudomonas cedrina* were allowed to form biofilms at 4 °C under continuous flow of nutrients in a CDC reactor (CBR 90; Biosurface Technologies, USA). The mature biofilms were treated with commercial enzyme cleaners, EnduroZyme (protease), DualZyme (protease and lipase), and TriZyme (protease, amylase, and cellulase). The dispersion with EnduroZyme was significantly ($p < 0.05$) higher than the other enzyme cleaners. Then the biofilms were treated with hot water and sodium hydroxide, and enzyme cleaners (sequential treatment). The cell counts after sodium hydroxide + Enzyme cleaners were below the detection limit. The microscopic observations with epifluorescence microscopy showed that the coupons had less fluorescence after the sequential treatment. FTIR observations showed that the extracellular polymeric substances (EPS) isolated after sodium hydroxide + enzyme cleaners differed from the untreated and sodium hydroxide-only-treated EPS. Biofilm regrowth was significantly ($p < 0.05$) lower in the biofilms treated with sodium hydroxide + EnduroZyme compared to acid-treated control coupons. The sequential treatment with sodium hydroxide and enzyme cleaners reduced the biofilm footprints, representing a better clean than enzyme treatment alone or sodium hydroxide-only cleaning.

1. Introduction

Pseudomonads are psychrotrophic, motile, aerobic, gram-negative rods. Pseudomonads produce various thermostable enzymes and pigments that affect the quality of dairy, poultry, and meat products (Raposo et al., 2016). The common psychrotrophic pseudomonads isolated from the cold chain are *Pseudomonas fragi*, *P. lundensis, P. fluorescens,* and *P. putida* (Nychas et al., 2008). Pseudomonads are strong biofilm formers at cold temperatures, with the extracellular polymeric substances (EPS) made up of polysaccharides, proteins, eDNA, and lipids (Flemming & Wingender, 2010). The cold stress encourages the overproduction of the EPS matrix of pseudomonads and results in strong biofilm formation (Liu et al., 2023). The EPS produced by pseudomonads protects the biofilm cells from adverse conditions and antimicrobials (Flemming & Wingender, 2010).

Pseudomonads form biofilms at the air-liquid interface; more access to oxygen encourages their biofilm formation (Ardré et al., 2019). In food processing environments, partly filled utensils, silos, tankers, and stagnated water represent the air-liquid interface where pseudomonads form robust biofilms (Jha et al., 2020). The biofilm formation at the meniscus and air-liquid interface is common among environmental pseudomonads (Robertson et al., 2013). When colonising at the air-liquid interface, it provides access to both gaseous (oxygen) and liquid (nutrients) phases, a benefit to colonise in water-filled environments (Spiers et al., 2003).

The biofilm elimination strategies for pseudomonads, such as sanitizers (Li et al., 2022b; Liu et al., 2023), cleaning chemicals (Shah & Muriana, 2021), quorum quenching molecules (Khalid et al., 2022), and cold atmospheric plasma (Lavrikova et al., 2025), target the biofilm cells, and the remaining biofilm EPS on the surface can attract bacteria to recolonise the surface. This remaining EPS on the surface is known as biofilm footprints (Neu & Marshall, 1991). There are very few strategies targeting the EPS matrix, which includes cold plasma, ultrasound, and enzyme cleaners. Enzymatic inactivation is the most feasible among those strategies that target the EPS. However, the dispersed cells can still be viable and cause biofilm formation of dispersed aggregates on a

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different surface, and it cannot be considered as a complete removal (Wang et al., 2023).

This study focuses on eliminating both biofilm cells and EPS in the air-liquid interface and submerged systems. This study sequentially treated the biofilms of psychrotrophic pseudomonads with hot water and sodium hydroxide, followed by commercial enzyme cleaners. The effects of enzyme cleaners on the biofilm footprints were evaluated by FTIR, microscopy, and biofilm regrowth.

2. Materials and methods

2.1. Bacterial isolates

Strong biofilm-forming isolates at cold temperatures, 3SM (*P. lundensis*) and 20SM (*P. cedrina*), were chosen for this study. The isolates were recovered from chilled milk tankers across New Zealand. The biofilm formation ability at cold temperatures was confirmed by the crystal violet assay. The identifications were compared using 16S rDNA sequencing.

2.2. Biofilm formation

Biofilms were allowed to form on a CDC reactor (CBR 90; Biosurface Technologies, USA) on stainless-steel coupons (304 grade with 2B finish with a total surface area of 2.26 cm^2) under continuous flow of half-strength Trypticase Soy Broth (TSB, Difco™, Becton and Dickinson company, USA) as a growth medium at a flow rate of 3.3 mL/min. The isolates were inoculated in TSB for 24 h to reach the OD600 of 0.05 ± 0.15. Two per cent (2 %) of this culture was added directly to the reactor beaker containing 400 mL of half-strength TSB (approximately 3 log CFU/mL). The reactor was run under turbulent flow at a Reynolds number of 4129. The reactor was assembled, autoclaved, and run for over a week at 4 °C. The coupon holders were lifted to create an air-liquid interface on the top coupons, and the remaining coupons were submerged. The top coupon on the air-liquid interface biofilms on its surface is denoted as A-L, while the two submerged coupons in the liquid are noted as L1 and L2. These 168-h-old biofilms were used for further experiments (Prabhukhot et al., 2024).

2.2.1. Cell counts

The coupons were removed from the reactor, washed with 0.85 % physiological saline, and transferred to the vials containing 900 μL of saline. After transferring the coupons, 1g of sterile glass beads was added, and the vials were sonicated for 5 min. After sonication, the coupon-containing vials were vortexed vigorously to remove the cells from the coupons. One hundred microlitres (100 μL) of the contents from the vials were serially diluted and plated on the TSA (TSA, Difco™, Becton, Dickinson and Company, USA) plates. The viable bacterial colonies were counted and expressed as log CFU/cm^2.

2.3. Expression of EPS producing genes

2.3.1. Isolation of biofilm cells

The coupons were removed after 168 h of incubation. Eight coupons for each A-L and L biofilm were added to the vials containing 4 mL of sterile distilled water and 1g of glass beads. The contents were transferred into four 1 mL Eppendorf tubes. The tubes were centrifuged at 12000 g for 5 min at 4 °C. The supernatant was discarded, and the cell pellets were collected and added to a single Eppendorf tube. Planktonic cultures grown at 4 °C for 18 h were centrifuged at 12000 g for 5 min at 4 °C to collect the cell pellets (Huang et al., 2022).

2.3.2. RNA extraction

The RNA was extracted using the Nucleospin RNA plus kit (with gDNA removal column) (Machery-Nagel, Germany) according to the manufacturer's instructions. The cell pellets were lysed with lysis buffer, washed, and isolated in sterile RNAse-free water. The concentrations and purity were checked using a Genovonano spectrophotometer (Huang et al., 2022).

2.3.3. Real-time -quantitative PCR(RT-qPCR)

Real-time quantitative PCR was performed using the Light Cycler 480 (Roche Diagnostics, USA) to analyse the difference in EPS producng gene expression between the air-liquid interface and submerged biofilm cells. The primers used in this study are listed in Table 1. The qPCR was performed using the Luna ® Universal One-step RT-qPCR kit (New England Biolabs, USA) according to the manufacturer's instructions. The cycling conditions were as follows: reverse transcription of RNA to cDNA at 55 °C for 10 min, followed by initial denaturation at 95 °C for 1 min; the amplification was performed with 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s and extension at 60 °C for 30 s (Huang et al., 2022). The relative gene expression analysis was performed using the $2^{-\Delta\Delta Ct}$ Livak method. The data shown in this study were indicated as fold changes compared to planktonic cells.

2.4. Treating the biofilms with enzyme cleaners

EnduroZyme, DualZyme, and TriZyme were the enzyme cleaners from IXOM, Australia. The composition, temperature, and pH for optimal activity are provided in Table 2.

The 168 h biofilms of psychrotrophic pseudomonads on the stainless-steel coupons were removed and washed with sterile distilled water. The enzymes were prepared according to Table 2 in sterile distilled water. 5 mL of the prepared enzymes were poured into the wells of the 6-well polystyrene plates (FALCON ®, Corning Incorporated, USA). The coupons were immersed in the enzyme cleaners for an hour and incubated at 50 °C. After an hour, the coupons were washed with sterile distilled water and added to the vials containing 1 mL of saline. The coupons were mixed by vortex with glass beads to remove the biofilm cells. The mixture was serially diluted in sterile saline (0.85 %) and plated on Tryptic Soy Agar (TSA, Difco™, Becton and Dickinson company, USA) on TSA and incubated at 30 °C for 24 h to count colonies.

2.5. Treating the biofilms with sodium hydroxide and enzyme cleaners

After 168 h of incubation, the CDC reactor was cleaned using hot water at 55 °C and NaOH at 70 °C. The coupons containing holders were taken out and inserted into the beakers containing enzyme cleaners and incubated at 50 °C for an hour. The remaining cells were removed from the stainless-steel coupons, and bacteria were counted as described previously.

2.6. Microscopical observations

The coupons were stained with acridine orange (5 mg/mL, Acridine

Table 1
Primers used for quantification of EPS producing genes.

Genes	Sequence	Product size (bp)	Reference
pel A	F 5′-AAGAACGGATGGCTGAAGG-3′ R 5′-TTCCTCACCTCGGTCTCG-3′	250	(Ghafoor et al., 2013)
psl A	F 5′-ATCAATATCCGCTCCACGC-3′ R 5′-CTGCTGCTCTTCCCCCAGT-3′	140	Liu et al. (2023)
bcs A	F 5′-GATTTTCGACTGCGACCACGTS-3′ R 5′- ACATGTCGTTRCCRTCCTGCAC-3′	150	(Gao et al., 2017)
alg K	F 5′ -ATGCCTATGTATTCAGCCAAC-3′ R 5′-ATTCCTCGCCGTCTTCTTC-3′	200	Liu et al. (2023)
16S rRNA	F: 5′-GCCCCTGGACAAAGACTGAC-3′ R: 5′CATGGTTTACGGCGTGGACTACC-3′	90	Liu et al. (2023)

Table 2
Enzyme cleaners used in this study.

Product	Composition	Temperature	Concentration	pH
Superflux EnduroZyme	Protease	50 °C	0.5 % v/v	11
Superflux Dual Zyme	Protease and Lipase			10
Superflux Tri Zyme	Protease, cellulase, and amylase			10

Orange stain, BDH, England) for the epifluorescence microscope. The coupons containing biofilms were washed with sterile distilled water and air dried. One hundred microlitres (100 μL) of acridine orange was added to the surface for 3 min. The coupons were washed and air-dried. With an epifluorescence microscope (Nikon Eclipse Ni-L, Nikon Instruments, USA), the coupons were viewed with a TRITC filter (Excitation 532–550 nm and emission 574 nm). The images were captured by NIS Elements D-software (Version 6.02.01 (Build 1955), Nikon Instruments, USA).

2.7. Attenuated Total Reflection- Fourier Transform Infrared Spectroscopy (ATR-FTIR)

After cleaning with cleaners, the coupons were washed with sterile distilled water. The coupons were added to the vials containing 2 mL of sterile distilled water. The mixture was sonicated for 15 min at room temperature. The mixture was centrifuged to get rid of the cells and filtered through syringe filters to obtain cell-free EPS. The EPS was isolated in the same way from the untreated and the coupons treated with sodium hydroxide. The isolated EPS was freeze-dried and observed using FTIR with the following parameters: acquisition range 2000–400 cm^{-1}, scanning times of 32 s, and resolution 4 cm^{-1} (Li et al., 2022b). The acid-treated coupons were used as a negative control.

2.8. Regrowth after NaOH + enzymatic cleaners

The biofilms were grown for a week. The CDC reactor was flushed with hot water at 55 °C, NaOH at 70 °C, at a Reynolds number >12000. The EnduroZyme enzyme was chosen for this experiment. The enzyme cleaner was flushed (Reynolds number >12000) into the CDC reactor at 50 °C for an hour. Finally, the entire coupon containing holder system was briefly submerged in the hot water at 72 °C to remove any adhering cells. Four holders containing 3 coupons each (NaOH + EnduroZyme treated coupons) from the CDC reactor were retained, and four holders containing acid-treated coupons (Control coupons) were added. All holders with the baffle were transferred into a new CDC reactor beaker and allowed to run for 24 h. After 24 h, the isolates of 3SM and 20SM were inoculated. The regrowth was monitored up to 72h. The cell counts from the control clean coupons and NaOH + EnduroZyme cleaned coupons were compared to determine the effects of footprints left after NaOH + EnduroZyme treatment.

2.9. Data analysis

All the experiments were performed with three biological and three technical replicates. One-way analysis of variance (ANOVA) was generated to evaluate significant differences among the variables using Tukey's test, which had a p-value below 0.05 and was considered statistically significant. Data analysis was implemented using SPSS statistical software (Version 29.0.2.0; IBM®, USA). FTIR data were processed using Origin Pro software (Origin Pro 2025, 10.2 Origin Lab Corporation, USA).

3. Results

3.1. Biofilm growth

The biofilms on A-L coupons showed significantly ($p < 0.05$) higher cell counts than L1 and L2 coupons for both isolates (Fig. 1). The cell counts on the A-L coupons were 7.47 ± 0.21 (3SM) and 7.26 ± 0.25 log CFU/cm^2 (20SM). Introducing the air-liquid interface in the CDC reactor resulted in approximately a log CFU/cm^2 between the coupons.

3.2. Expression of EPS producing genes

The RNA extracted from L2 was low and not sufficient for qPCR. Four EPS producing genes were compared between the A-L and L1 biofilm cells (Fig. 2). The capsular polysaccharide alginate-producing genes were highly expressed in the A-L biofilm cells. The aggregative polysaccharide cellulose-producing genes were also highly expressed in the air-liquid interface biofilm cells. There was no significant ($p > 0.05$) difference between the expression of another aggregative polysaccharide-producing gene, *pel*, between the air-liquid interface biofilm cells and submerged biofilm cells. *psl* A-producing genes were highly expressed in the air-liquid biofilm cells. However, the expression of *alg* K and *bcs* A was significantly ($p < 0.05$) higher than *psl* A.

3.3. Treating the biofilms with enzyme cleaners

From 48 h to 72 h, the appearance of the air-liquid interface was observed on the A-L coupons. After a week of incubation in the CDC reactor, the cell counts in the A-L coupons reached the following cell counts in log CFU/cm^2:8.1 ± 0.14(3SM) and 7.89 ± 0.12 (20SM), and the L1 coupons reached 6.23 ± 0.11 (3SM) and 6.43 ± 0.28 (20SM). The cell counts from the L2 coupons were (log CFU/cm^2) 4.98 ± 0.21 (3SM) and 4.57 ± 0.16 (20SM) (Figs. 3A and 3B). When comparing the results of the enzyme cleaners, EnduroZyme significantly ($p < 0.05$) reduced the cell counts compared to the other two enzymes. The log reductions observed after treating with EnduroZyme were 4.21 ± 0.36 and 3.79 ± 0.35 log CFU/cm^2 for the A-L coupons, 3.29 ± 0.16 and 3.4 ± 0.19 log CFU/cm^2 for L1 coupons, and 2.24 ± 0.11 and 2.4 ± 0.25 log CFU/cm^2 for the L2 coupons. The log reductions for DualZyme were 1.45 ± 0.33, 1.42 ± 0.10, and 1.55 ± 0.31 log CFU/cm^2 for A-L, L1, and L2 of isolate 3SM and 1.93 ± 0.12, 1.89 ± 0.25, and 1.13 ± 0.28 log CFU/cm^2 for A-L, L1, and L2 of isolate 20SM. After treating with Tri Zyme, the log reductions were 1.76 ± 0.24, 1.35 ± 0.10, and 1.52 ± 0.18 log CFU/cm^2 for the isolate 3SM and 1.9 ± 0.18, 1.85 ± 0.25, and 1.2 ± 0.24 log CFU/cm^2 for the isolate 20SM (Fig. 3A and 3B). The log reduction was reflected in the microscopic observations. When the biofilms of isolates

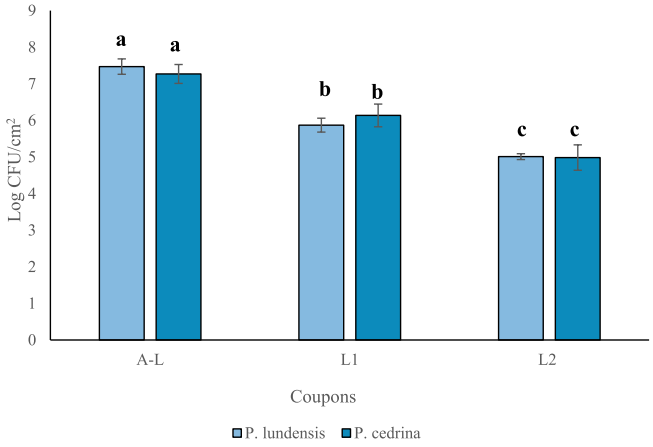

Fig. 1. Cell counts from A-L, L1, and L2 coupons after 168 h of biofilm formation. Different letters above indicate significant differences ($p < 0.05$).

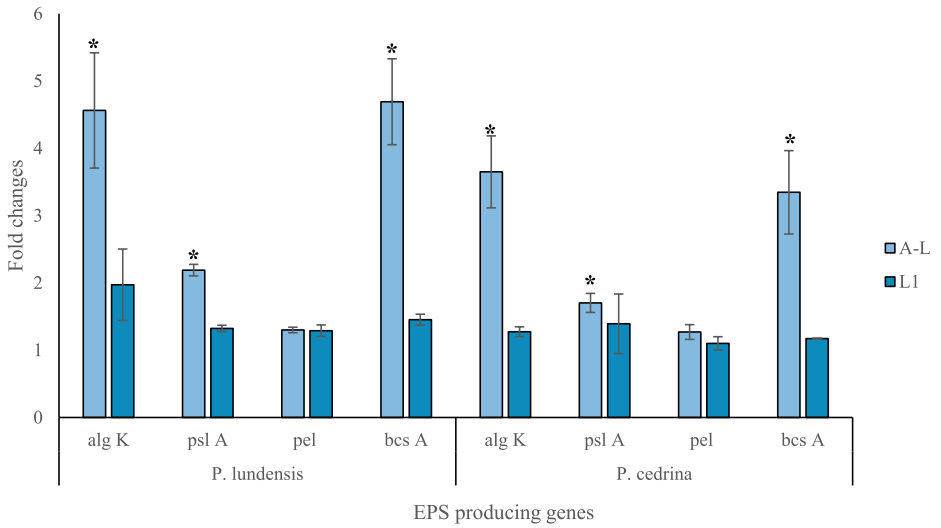

Fig. 2. Gene expression differences in EPS producing genes between air-liquid interface and submerged biofilm cells. "*" indicates significant differences ($p < 0.05$).

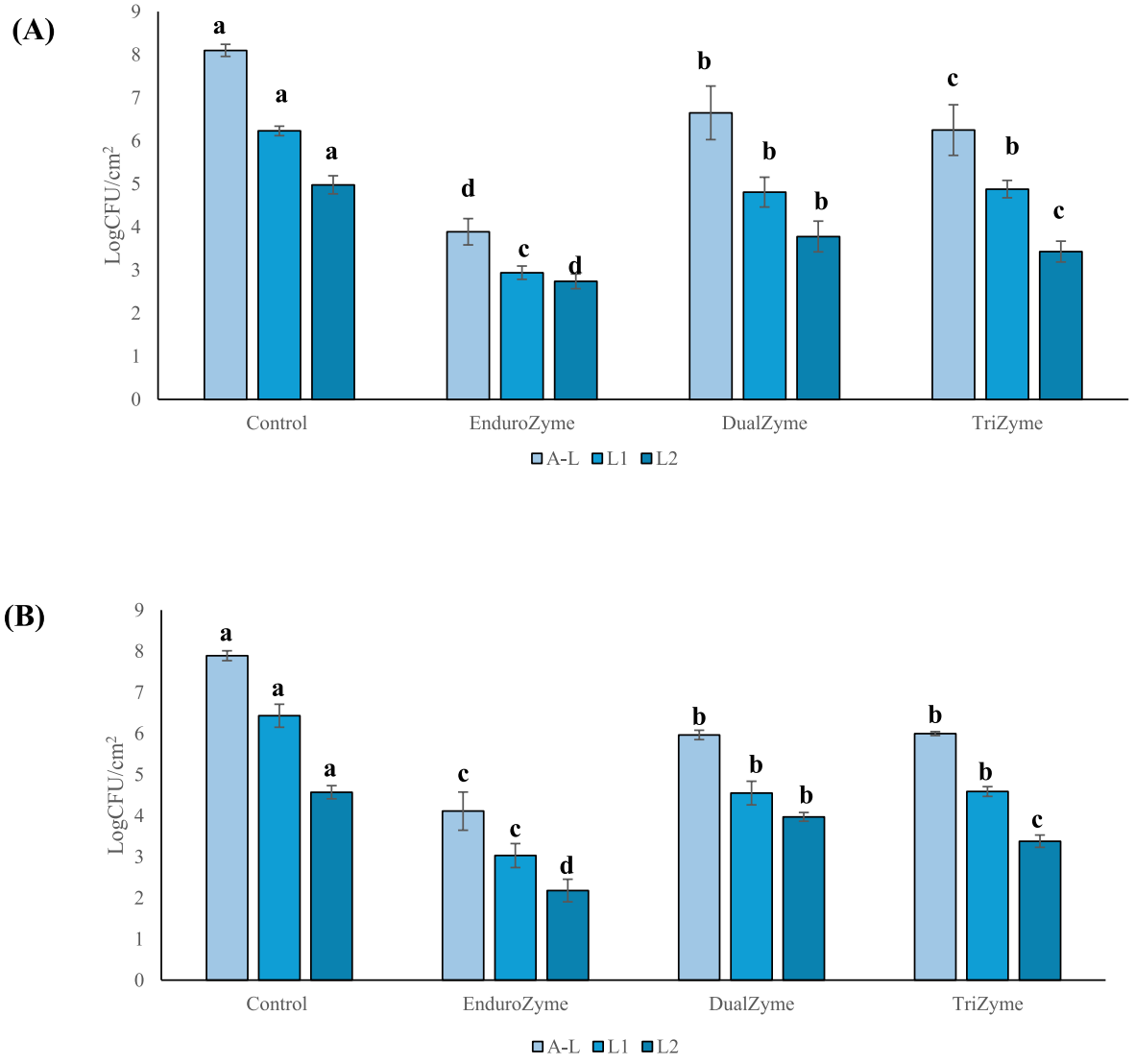

Fig. 3. (A) Cell counts of biofilms formed by isolate 3SM after treating with commercial enzyme cleaners, (B) Cell counts of isolate 20SM biofilms after treating with commercial enzyme cleaners. All the results were expressed as mean ± standard deviation. Different letters above each bar indicate significant differences ($p < 0.05$).

were treated with the enzyme cleaners, the enduroZyme-treated coupons showed more dispersed cells and fewer aggregates on the A-L coupons than the untreated coupons (Figs. 4A and B). The coupons treated with DualZyme still had the biofilms with full integrity in the A-L coupons. The L1 and L2 coupons showed aggregates rather than dispersed cells (Figs. 4C and D). The coupons treated with TriZyme had the biofilm structure and big aggregates of cells in the A-L, L1, and L2 coupons (Figs. 4E and F). The cell reduction from coupons treated with TriZyme and DualZyme was not significantly ($p < 0.05$) different and resembled the same with microscopic observations. Among the three enzyme cleaners, EnduroZyme resulted in approximately a 4-log reduction and the breakdown of the biofilm structures.

3.4. Sodium hydroxide + enzyme cleaners

From section 3.1, the biofilms did not disperse after being treated with enzyme cleaners, even though the EnduroZyme reduced the cell counts by approximately 4 log CFU/cm^2 for the A-L coupons. When the enzyme cleaners were applied after sodium hydroxide cleaning, the cell counts reduced below the detectable limit (1.7 log CFU/cm^2).

The epifluorescence microscope images showed that there was some fluorescence left on the coupons treated with NaOH + DualZyme and NaOH + TriZyme (Fig. 5C, D,5E, and 5F). The coupons treated with NaOH + EnduroZyme were clear for both isolates (Figs. 5A and B). From this, NaOH + enzyme cleaners removed biofilm EPS footprints effectively compared to treating with enzyme cleaners alone.

3.5. ATR-FTIR observations of EPS after treatment with enzyme cleaners

The EPS isolated after NaOH and NaOH + enzymes were observed under FTIR. A sterile stainless-steel coupon was used as a control. EPS isolated from the untreated biofilms was used to compare the remaining debris after NaOH and NaOH + enzymes. The amide I and amide II bands at 1650 and 1540 cm^{-1} were present in the untreated EPS. There was a less prominent peak of this wavelength present in the EPS isolated after NaOH cleaning (Figs. 6A and B). The peak at 1055 cm^{-1} represents the polysaccharides. This peak is still present in the EPS isolated after NaOH. The EPS isolated after NaOH + enzymes showed the absence of the polysaccharide peaks, but there was a presence of a single protein peak (Figs. 6A and B). However, this peak was different from the one present in untreated samples. The EPS isolated after NaOH + enzymes was not as clear as the control stainless steel. The enzymes used in the cleaning solutions might be responsible for the protein peaks. This suggests that the NaOH + Enzymes leave enzyme debris after cleaning.

3.6. Regrowth

There was no appearance of an air-liquid interface for both control coupons and NaOH + EnduroZyme-treated coupons. The cell counts on the control coupons were significantly higher than the cell counts of coupons treated with NaOH + EnduroZyme for both the isolates (Figs. 7A and B). The highest cell counts reached by isolate 3SM were 5.48 log CFU/cm^2 at the control A-L coupon (72h), while the highest cell count for NaOH + EnduroZyme-treated coupons was 4.84 log CFU/cm^2 at the A-L interface at 72 h (Fig. 7A). For the isolate 20SM, the highest cell count reached by the control A-L coupons at 72 h was around 5.72 log CFU/cm^2 and the A-L NaOH + EnduroZyme treated coupon showed highest cell count at 72h which was 5.30 log CFU/cm^2 (Fig. 7B). Compared to the isolate 3SM, 20SM established biofilms on NaOH + EnduroZyme-treated coupons with cell counts closer to the control coupons. The NaOH + EnduroZyme-treated coupons showed lower cell counts might be due to the adhering enzyme cleaner on the surface of the coupons, which prevented the initial adhesion of the biofilm cells.

4. Discussion

Biofilm formation at cold temperatures is important, as most of the perishable food processing environments opt for temperatures between 4 and 7 °C. Pseudomonads are psychrotrophic bacteria and form strong biofilms with robust EPS production at cold temperatures (Wickramasinghe et al., 2020; Liu et al., 2023). This present study focused on the biofilm formation of psychrotrophic pseudomonads at 4 °C and highlighted that EPS production was highest at the air-liquid interface.

The *B. cereus* biofilm cells yielded similar cell counts on both submerged and semi-submerged biofilms formed on a stainless-steel surface under static conditions (Ren et al., 2024). However, with pseudomonads under a continuous system, the cell counts were significantly higher on the A-L coupons than on L1 and L2.

This study, for the first time, compared the gene expression between the air-liquid interface and submerged biofilm cells. Studies found that the EPS producing genes in pseudomonads, such as *alg K* and *psl A,* are highly expressed at 4 °C, compared to 25 °C (Liu et al., 2023; Vásquez-Ponce et al., 2017). In this present study, although both biofilm cells were grown at 4 °C, there was a difference in the expression of EPS producing genes. The genes *alg K*, *psl A*, and *bcs A* were highly expressed in air-liquid interface biofilm cells compared to submerged biofilm cells.

There was no difference between the expression of *pel* genes. Pellicle formation of these isolates was observed under static conditions (Data not shown). In the CDC reactor, the pellicle formation was not observed. Comparing the *pel* gene expression between the static and continuous systems is needed.

A study comparing the combination of enzyme cleaners against biofilms grown on PET and SS coupons showed that the highest removal percentage obtained was 82.9 % by the formulation that consisted of amylase, protease, and lipase for the SS surface. Meanwhile, for the PET surface, the highest removal percentage was 82.6 % for the same combination. (Tsiaprazi-Stamou et al., 2019). In this present study, compared to the combination of amylase, protease, cellulase (TriZyme), and protease and lipase (DualZyme), protease (EnduroZyme) alone effectively removed the biofilms. The enzyme cleaners could not disperse the biofilms completely. The microscopic observations showed similar results, more dispersion with EnduroZyme than with the other two enzymes. However, this present study was focused on the pseudomonad biofilms, and the above-mentioned study was from the meat isolates, and different biofilms respond differently to the enzyme cleaners.

In this present study, when the biofilms were treated with NaOH and enzyme cleaners, the remaining cells were below the detectable limit (1.7 log CFU/cm^2). In a study by Iñiguez-Moreno et al. (2021), when the alkaline protease and α-amylase (180 Modified Wohlgemuth unit/g for 30 min at 25 °C) were applied to the multispecies biofilms grown on the stainless-steel coupons, this resulted in 2–3 log CFU/cm^2 reductions. However, when the enzyme treatment is combined with PAA (Peracetic Acid, 200 mg/L for 10 min at 25 °C) and SBC (Sanicip Bio Control, 30 g/L for 30 min at 25 °C), the log reduction was around 6.5 log CFU/cm^2. From these observations, the application of enzyme cleaners with sanitizers or other cleaning agents is needed for effective cell reduction and possible colonization of dispersed cells.

In this present study, the sequential treatment of caustic and enzyme cleaners resulted in high log reduction and EPS footprint removal. In another study on multispecies biofilm removal, sequential treatment steps included alkali, surfactant 1, acid, enzyme, surfactant 2, and sanitizers (Singh & Anand, 2022). Among the tested bacteria in the multispecies consortia, around 1.13 ± 0.03 log CFU/cm^2 *Bacillus* sp cells were remaining was difficult to eliminate. This sequential CIP treatment eliminated the *Enterococcus* sp and *Escherichia coli*, but *Staphylococcus* sp, *Klebsiella* sp, and *Corynebacterium* sp remained (0.05, 0.010, and 0.05 log CFU/cm^2). This observation also highlighted the use of sequential treatments in biofilm removal (Singh & Anand, 2022).

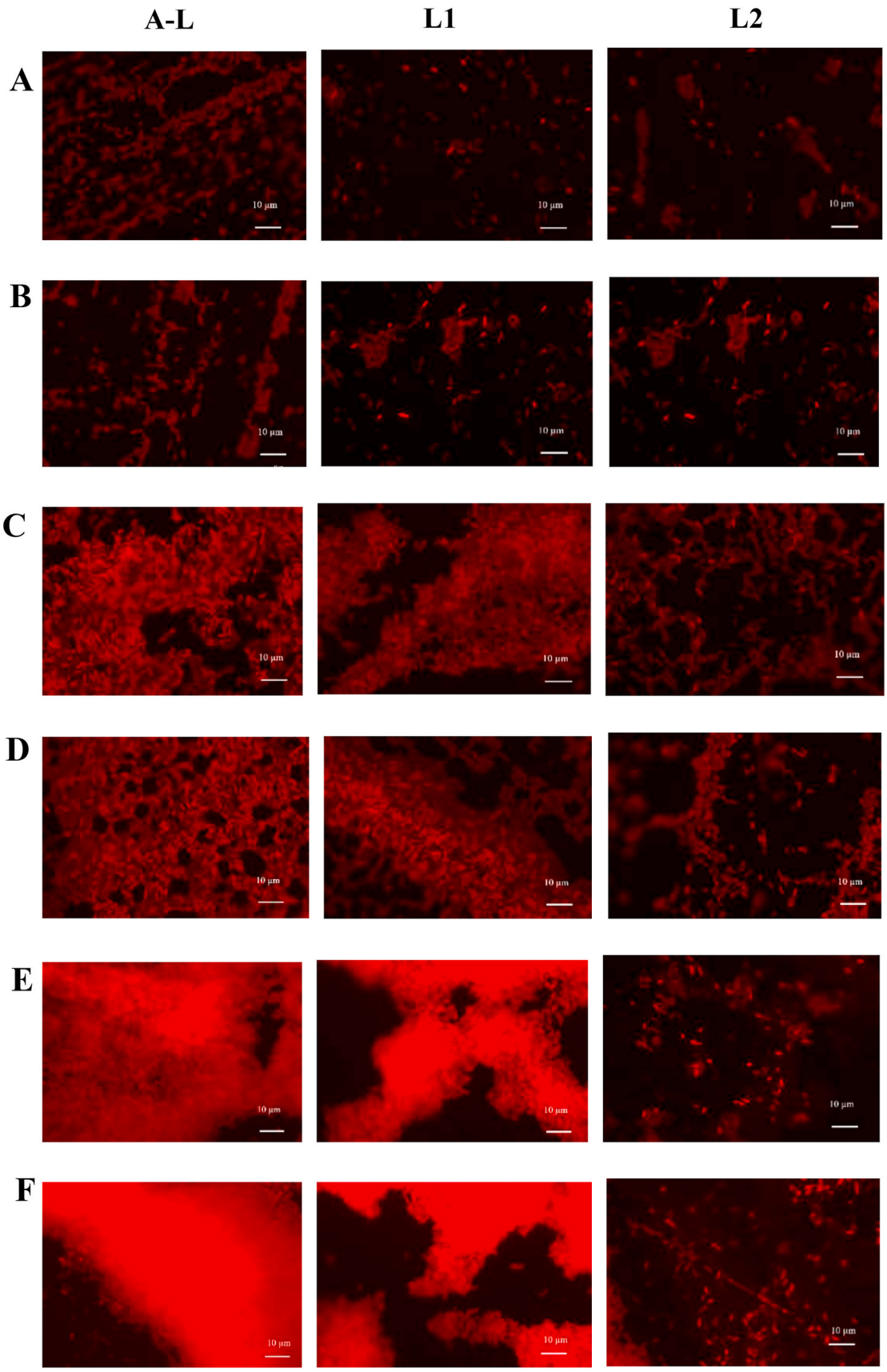

Fig. 4. (A) & (B) EnduroZyme-treated coupons of isolate 3SM and 20SM, (C) & (D) DualZyme-treated coupons of isolates 3SM and 20SM, (E) & (F) TriZyme-treated coupons of isolates 3SM and 20SM.

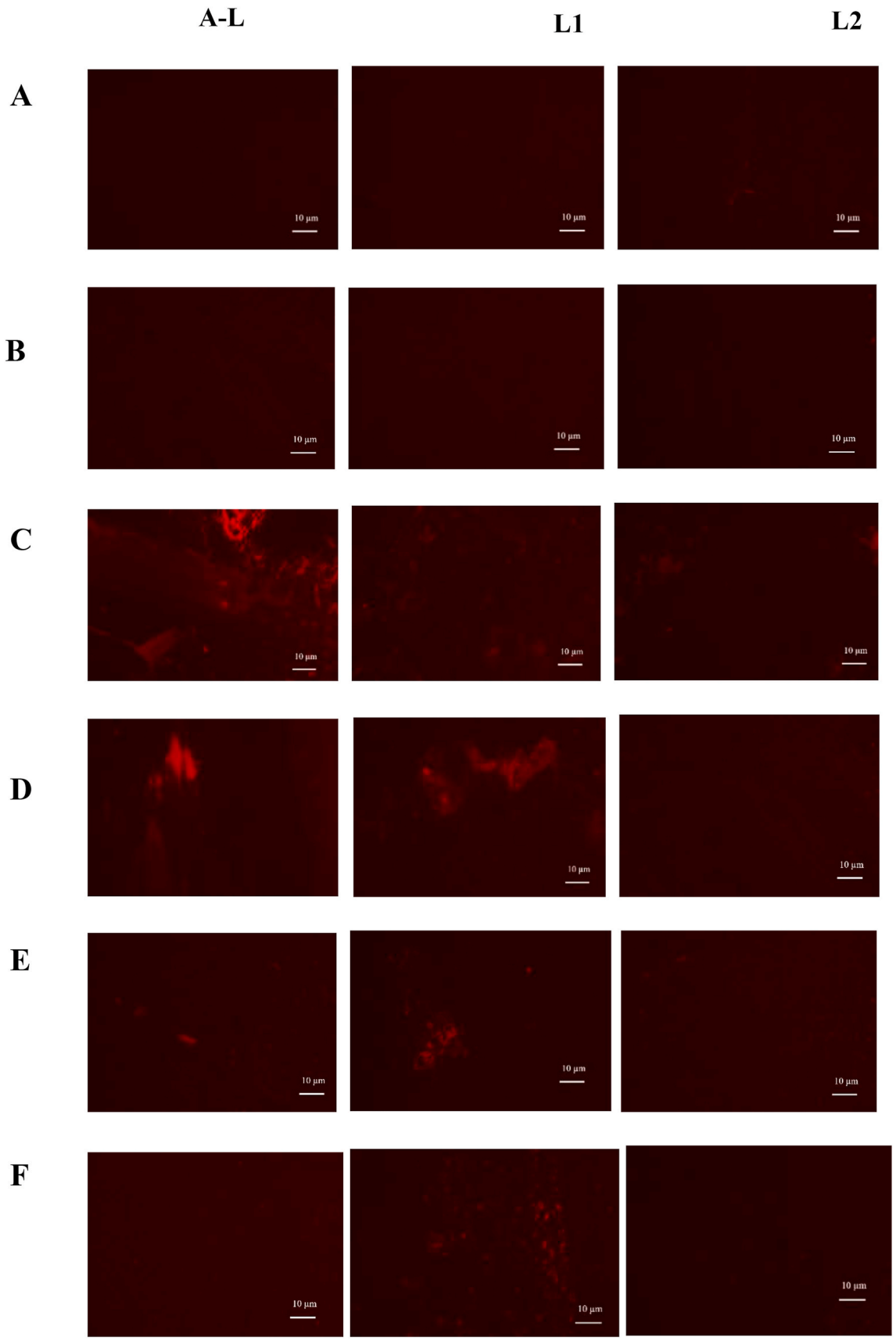

Fig. 5. Epifluorescence microscopic images of (A) & (B) NaOH + EnduroZyme treated coupons of isolates 3SM and 20SM, (C) & (D) NaOH + DualZyme treated coupons of isolates 3SM and 20SM, (E) & (D) NaOH + TriZyme treated coupons of isolates 3SM and 20SM.

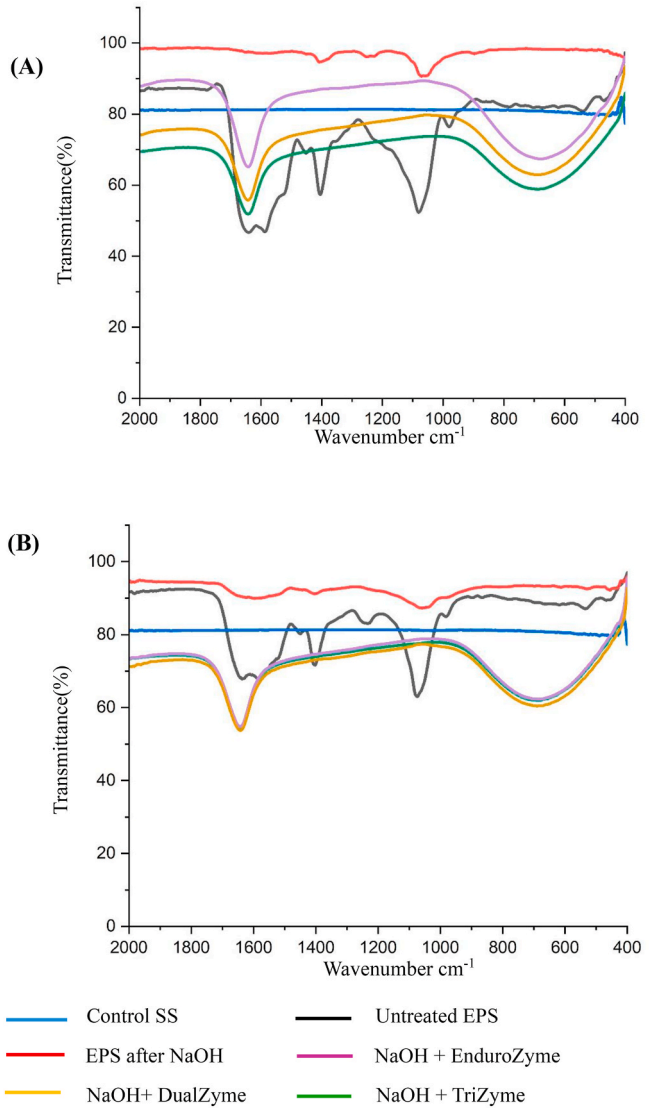

Fig. 6. ATR-FTIR images of untreated, NaOH, and NaOH + Enzyme treated coupons of isolates (A) 3SM and (B) 20SM.

In this present study, the NaOH-treated EPS showed reduced intensities in protein and polysaccharide peaks compared to the untreated EPS. However, the FTIR observations of the EPS isolated after NaOH + Enzyme cleaners showed different peaks from the untreated EPS and NaOH-treated EPS. This data suggests that the NaOH + Enzyme cleaners removed the biofilm footprints. The different peaks resulted from the enzyme cleaners, which were confirmed by the FTIR observations of the enzyme cleaners (supplementary file 1). In a study by Li et al. (2022b), the FTIR observations of *P. aeruginosa* biofilms treated with DDAB (Didecylmethylammonium bromide) and SAEW (Slightly acidic electrolysed water) showed reduced intensities compared to the untreated biofilms.

In this present study, EnduroZyme (Protease-subtilisin) was effective among the three enzyme cleaners and resulted in around 4 log CFU/cm^2 reduction at the A-L coupons. The coupons treated with EnduroZyme alone showed dispersed cells. When combined with NaOH treatment, there is no debris when observed under an epifluorescence microscope. The Dual Zyme (Protease and lipase) and TriZyme (Protease, amylase, cellulase) showed some debris, which can be seen with epifluorescent microscopic images (Fig. 4C-F). The SEM observations showed no live cells after treating the biofilms with CIP and enzyme cleaners. The role of NaOH here was to remove the cells, and the enzyme cleaners removed the EPS. This sequential application of NaOH and enzyme cleaners resulted in cell death and EPS reduction. The DDAB and SAEW combined treatment removes the biofilm structures of *P. aeruginosa,* and some of the small cell clusters remain on the surface (Li et al., 2022b). With a confocal microscope, the authors observed the reduction in green and blue fluorescence, which indicates the reduction in the EPS (Li et al., 2022b).

The NaOH + EnduroZyme-treated coupons showed no debris and were chosen for the regrowth experiment. After the NaOH + EnduroZyme treatment, the new inoculum was introduced, and the regrowth of the negative control and NaOH + EnduroZyme-treated coupons was compared. The NaOH + EnduroZyme-treated coupons resulted in significantly ($p < 0.05$) fewer cells compared to the control acid-treated coupons. The FTIR observations showed peaks other than EPS for the NaOH + Enzyme-treated coupons. This indicates the presence of enzymes on the surface. However, it was not seen on the microscopic images. These enzymes could have prevented more cells from attaching to the NaOH + enzyme-treated coupons, resulting in lower cell numbers.

In a work by Li et al. (2022a), the coating of proteinase-K (45U/mL) on the stainless-steel surface inhibited the biofilm formation by 58.6 %. The difference between the control and enzyme-treated coupons was less than 2 log CFU/cm^2. The enzyme coating was not enough to prevent adhesion and biofilm formation completely (Li et al., 2022a). In this present study, the log difference between the control and NaOH + EnduroZyme-treated coupons was around 0.5 to 1 log CFU/cm^2 for the isolate 3SM and 0.5 log CFU/cm^2 for the isolate 20SM. The enzyme cleaners can prevent biofilm formation. However, the interference of remaining enzymes on the food matrix needs to be considered.

In this present study, the cells were eliminated by NaOH and pasteurisation steps, and the remaining EPS footprints were eliminated by enzyme cleaners, resulting in less aggressive biofilm formation. Even after being treated with enzymes, the A-L coupons yielded higher cell counts than L1 and L2, indicating the importance of the air-liquid interface in the biofilm formation of pseudomonads. In a study by Cervantes-Huamán et al. (2025), the *C. cassia* essential oil combined with protease and amylase resulted in cell numbers lower than 1.45 log CFU/cm^2 for *Listeria monocytogenes* and *Salmonella* Typhimurium strains. However, the regrowth of these strains was more aggressive, suggesting the remaining debris encouraged the regrowth (Cervantes-Huamán et al., 2025).

This present study demonstrated that combining the strategies that target both cells and EPS would be effective in complete biofilm removal. However, this present study did not include the cost analysis of the proposed sequential treatment. Cost effectiveness, safety on food matrices, and environmentally friendly design are the other important factors to be considered while designing the biofilm removal strategies.

5. Conclusion

The two strong biofilm-forming isolates, *P. lundensis* and *P. cedrina*, showed higher cell counts and expressed higher EPS producing genes at the air-liquid interface. The enzyme cleaners, when treated alone, cannot disperse the biofilms (both air-liquid interface and submerged) of these isolates completely. However, when treated after the NaOH cleaning and the enzyme cleaners reduce the remaining biofilm footprints at both the air-liquid interface and the submerged coupons. The regrowth of the biofilms of the isolates *P. lundensis* and *P. cedrina* on the NaOH + Enzyme-treated coupons is reduced, indicating the effectiveness of enzyme cleaners in removing biofilm footprints. The cost-effectiveness of these sequential treatments needs to be explored, and future studies should focus on cost-effective, environmentally friendly cleaners to completely remove the biofilms.

CRediT authorship contribution statement

Srinithi Muthuraman: Writing – review & editing, Writing –

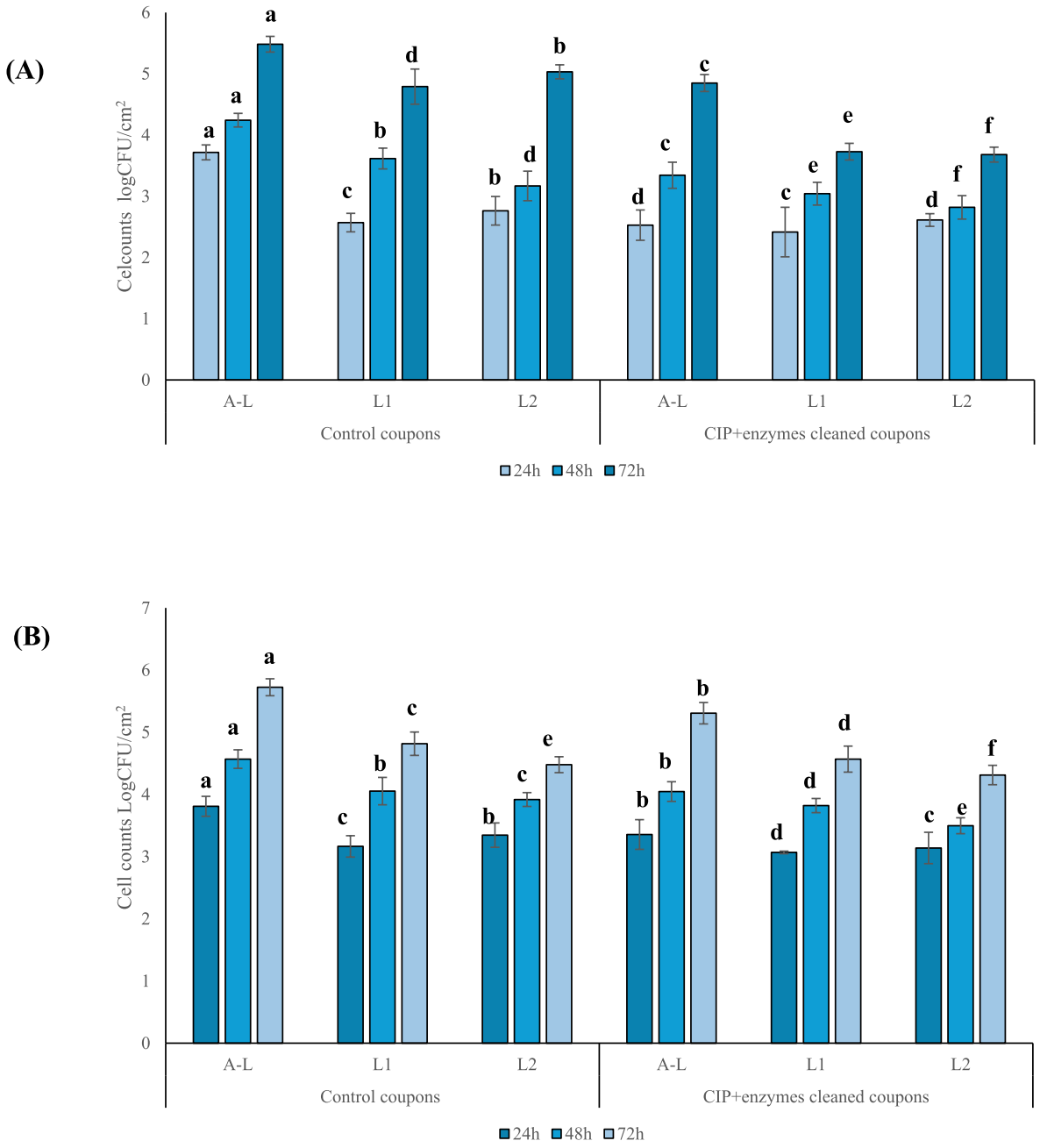

Fig. 7. Graph (A) shows the regrowth of isolate 3SM after CIP + EnduroZyme cleaning, while graph (B) shows the regrowth of isolate 20SM. All results were expressed as mean ± standard deviation. Different letters above each bar indicate significant differences ($p < 0.05$).

original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jon Palmer:** Writing – review & editing, Supervision, Conceptualization. **Steve Flint:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111858.

Data availability

Data will be made available on request.

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