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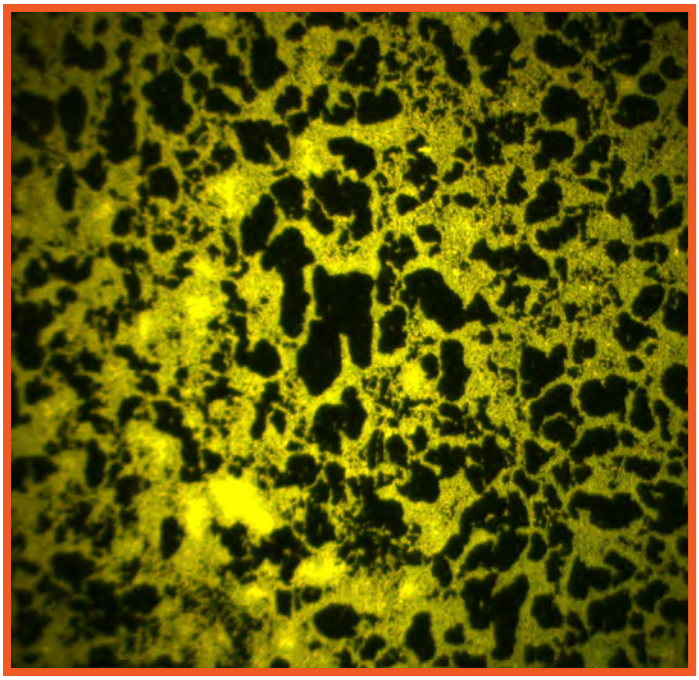

Food Grade Dye for Assessment of Biofilm Removal from Stainless Steel by Cleaning and Sanitizing Agents

ABSTRACT

The objective of this study was to evaluate a food grade dye, erythrosin B, for use in the development of a quantitative color difference methodology to measure the efficacy of cleaner/sanitizer solutions in removing biofilm components from stainless steel surfaces. Biofilms of *Listeria innocua* and *Pseudomonas putida* were grown on stainless-steel coupons, subjected to various cleaner/sanitizer treatments, and then stained with erythrosin B. Resultant coupons were photographed and mean L*a*b* color differences between background and dyed area evaluated. Color differences conformed to a scale correlated with human visual perception. Results indicated that the method provides sensitivity for visual appraisal of treatment-response as well as species-response relationships. The method shows potential as an enhancement for quantitative visual assessment of cleaning/sanitizing treatments of biofilms in a laboratory setting, and supplemental research is warranted to assess its efficacy for GRAS inspection of food processing environments as part of the Hazard Analysis Critical Control Point program.

INTRODUCTION

Microbiological contamination caused by inadequate design of equipment with regard to sanitation or ineffective sanitation procedures may lead to unacceptable risks in food production. Very low levels of contamination can lead to serious illness. In the case of *Listeria monocytogenes*, the lethal dose for humans has not been ascertained, but studies on mice suggest that ingestion of as few as 100 organisms can lead to abortion or death in pregnant or immunocompromised individuals *(7)*. Improperly cleaned surfaces may have organic soil or biofilm material attached, which can promote more soil buildup. The presence of water may contribute to the development of new bacterial biofilms that potentially contain pathogenic organisms *(4, 14, 18)*. When food passes over these contaminated surfaces, cross-contamination may occur *(2, 3, 5)*. In addition, the type of food contact surface and its topography play a role in the

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ease of decontaminating a surface *(6, 8)*. Abraded surfaces accumulate soil and prove more difficult to clean than smooth surfaces *(6)*.

In part because of the risks posed by improperly cleaned and sanitized surfaces, the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) established the Pathogen Reduction; Hazard Analysis Critical Control Point (PR/HACCP) Systems Final Rule *(19)*. Under this program, FSIS assesses industry performance and controls for reducing contamination in raw meat and poultry products, and processors must have and implement a sanitation standard operating procedure (SSOP) that addresses sanitation conditions and practices before, during, and after processing. An SSOP may include a pre-operational task during which selected areas of the

establishment and equipment that pose a high risk of contamination are organoleptically, primarily visually, inspected to assess sanitary conditions. Visual inspection may be of little value because the residues, whether of food or bacterial origin, may be present in quantities too small to be detected by the human eye, although they still present a contamination risk. A method to enhance the visual assessment or inspection of potentially contaminated areas in the processing line would therefore be of great value to processors in identifying and measuring contaminated areas in or on processing equipment as well as in identifying design flaws that make sanitation of equipment difficult.

Various dyes have been employed to monitor total biofilm biomass *(13, 17)*. Some of those most commonly used, such as crystal violet *(11, 16)* and safranin red *(12)*, offer simplicity and direct optical visualization. These dyes are positively charged and bind to the negatively charged surfaces of bacteria, both viable and non-viable, and to biofilm matrix components including carbohydrates, DNA and proteins. Although these dyes have been proven useful as screening tools for measuring the effectiveness of biofilm removal by various cleaning agents and sanitizers, they exhibit toxic properties and are not approved by FDA for use in food processing environments.

Erythrosin B, also known as FD&C Red #3, is an FDA-approved food coloring dye that has been utilized since the early 1960s as a dental plaque disclosing agent *(1)*. The dye has also found use as a colorimetric reagent for protein determination *(15)* because of its ability to form intensely colored complexes. In addition to its characteristic visible red color, the dye exhibits fluorescence in the visible region centered at 550 nm (green-yellow). This property has allowed erythrosin B to be utilized as a vital exclusion stain for determination of cell viability after lethal treatment *(9)*. These traits suggest that erythrosin B has potential use as a screening method for biofilm removal and in the visual determination of cleaning efficacy in food processing environments. The goal of the present study was to evaluate the potential for adapting erythrosin B for use as a non-specific dye for enhancing the visualization of biofilm removal from stainless steel in addition to monitoring removal of other organic soils potentially present in meat and poultry processing environments. Two organisms commonly associated with biofilm formation in food processing environments are *Listeria* and *Pseudomonas*. Although they do not generally occur as monospecies biofilms in nature, both are regularly used as biofilm surrogates for research purposes. *Listeria innocua* and *Pseudomonas putida* were chosen as surrogates in the present work, partly because of their non-pathogenicity and hence their safety.

MATERIALS AND METHODS

Cell attachment

Stock cultures of *L. innocua* and *P. putida*, obtained from the U.S. National Poultry Research Center culture collection, were inoculated into tryptic soy broth and incubated at 37°C for 24 h and then plated onto Brain Heart Infusion (BHI) agar (Oxoid GmbH, Wesel, Germany). Individual isolated colonies were taken from fresh cultures of *L. innocua* and *P. putida* to create lawns (plates entirely covered with growth) to make 10 lawn plates. Growth from these plates was transferred to 700 ml of PBS (phosphate buffered saline) with sterile swabs. Final inoculum concentrations were 9×10^8 CFU/ml for *L. innocua* and 2×10^8 CFU/ml *P. putida*. Stainless steel grade 316 L coupons having a 2B finish were cut to 2.5 × 7.5 cm dimensions. A batch of coupons was prepared by cleaning the surfaces with acetone, autoclaved, placed in a stainless-steel microscope slide staining rack, and immersed approximately halfway for 2 h into a slide tray containing the inoculated PBS broth at 25°C. The coupon rack was then rinsed three times with sterile deionized water and transferred to a second slide tray containing 700 ml of sterile 1/10 TSB (tryptic soy broth) medium (Becton Dickinson & Co., Franklin Lakes, NJ) at 25°C for 24 h, to promote cell attachment. After 24 h, the slides were rinsed with sterile water and put into petri dishes for temporary storage.

Cleaner and sanitizer treatments

Tween 80, α-amylase from *Bacillus licheniformis*, and 39% peroxyacetic acid were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Alcalase from *Bacillus licheniformis* was purchased from EMD Millipore (MilliporeSigma, Burlington, MA), and 30% non-stabilized hydrogen peroxide was purchased from Acros Organics (Acros Organics, Morris, NJ). Two commercial cleaning/disinfectant solutions were provided by their manufacturers. Following cell attachment, duplicate coupons were immersed in one of the eight treatment solutions, shown in *Table 1*, for 15 m. Following treatments, duplicate coupons were rinsed three times in deionized water. One of the duplicates was swabbed with a pre-moistened sterile swab, which was returned to the remainder of 1 ml PBS, vortexed, and subjected to a series of 1:10 dilutions for CFU/ml counts on BHI agar plates for

TABLE 1. Treatments evaluated for cleaning and sanitizing efficacy

Treatment	Description
NT	No treatment
H_2O	pH = 7.2 buffered DI water, 0.1% Tween 80
Enzyme	0.1% (v/v) amylase and alcalase, pH = 7.2 buffered DI water, 0.1% Tween 80
pH = 13	pH = 13 sodium hydroxide, 0.1% Tween 80
CS1	Commercial solution 1
CS2	Commercial solution 2
PAA	2000 ppm peroxyacetic acid, pH = 3.4, 0.1% Tween 80
HP	5% hydrogen peroxide, pH = 7.2 buffered DI water, 0.1% Tween 80

both *L. innocua* and *P. putida*. The second duplicate coupon was stained for analysis by visible color measurement and fluorescence microscopy. The treatment solutions were also subjected to 1:10 dilutions to determine counts. This process was repeated three times on three different days, using the same procedure with freshly prepared inocula and chemical solutions.

Dyeing methods

For dyeing with erythrosin B, biofilm coupons subjected to the various cleaning and sanitizing treatments were subsequently rinsed 3 times with deionized water. The non-ionic form of erythrosin B (CAS 15905-32-5, Sigma-Aldrich, St. Louis, MO) was dissolved in 50 ml isobutyl alcohol (Sigma Aldrich, St. Louis, MO), and following complete dissolution, 50 ml of propylene glycol was added, for a final concentration of 0.1 g/100 ml. The coupons were then laid flat and the entire surface covered with 1 ml of the erythrosin B dye solution for 5 min, following which the coupons were rinsed with deionized water and allowed to air dry.

Biofilm coupons subjected to the various cleaning and sanitizing treatments were dyed with a 0.5% solution of crystal violet dissolved in 80:20 water:methanol solution. Coupons were laid flat and the entire surface covered with 1 ml of the crystal violet solution for 5 min, followed by rinsing with deionized water and subsequent air drying.

Photographic conditions and image processing.

Images were captured using a Nikon D810 DSLR with a Micro-Nikkor 60mm f/2.8G lens mounted on a Beseler CS-14 copy stand (Stroudsburg, PA) providing an overhead view of the coupons. Indirect illumination was provided by two 50W 4700K Solux MR16 bulbs (Rochester, NY) positioned laterally to the coupons. Images were captured and processed in 14-bit uncompressed RAW format, using Adobe RGB color space. An in-camera custom white balance was set using a 5" × 5" Spectralon panel (SRT-75-050, Labsphere, North Sutton, NH). Additional camera settings consisted of a shutter speed of 0.5 s, an aperture of f/11, and a base ISO of 64. Mean L*a*b* values from the control and treated halves of the coupons were then determined by use of Adobe Photoshop CC. RAW images were converted to L*a*b* mode, and the rectangular selection tool was used to define the halves of each coupons. Mean L*a*b* values were then recorded. In each case, the difference in color, ΔE*, between the two halves of each coupon was calculated as described by McLaren *(10)*:

$$\Delta E^* = [(L1^*-L2^*)^2 + (a1^*-a2^*)^2 + (b1^*-b2^*)^2]^{1/2} \quad \text{Equation 1}$$

where 1 and 2 (within the parentheses) denote the two portions (biofilm and non-biofilm) of the coupons. Higher ΔE* values are defined as being more perceptible by the human eye, with values ≤ 1 generally denoted as equivalent.

Fluorescence microscopy and microspectrophotometry

Microscopic fluorescent images were collected with a Craic UVM-1 fluorescence microscope (Craic Technologies, Inc., San Dimas, CA). Images were obtained at 100× magnification with use of a 450–490 nm bandpass filter for excitation and a 515 nm long pass filter for emission. Fluorescence spectra of microscopic images were obtained from 450 to 900 nm, using a Craic MSP 10 spectrophotometer coupled to the microscope. Each spectrum was averaged over 10 scans, with a 1-sec integration time.

Statistical analysis

Three replications were conducted for each of the eight cleaner-sanitizer treatments (n = 3). Colony counts were $\log_{10}$ transformed and geometric means were analyzed by use

TABLE 2. Mean log CFU/cm², with standard deviation in parentheses, of *Listeria innocua* and *Pseudomonas putida* recovered from stainless steel coupons following no (NT), water (H_2O), and enzyme treatments. Pairwise multiple comparisons by Holm-Sidak method* (n = 3)

Treatment	*Listeria innocua*	*Pseudomonas putida*
NT	6.08 (0.35) a	3.95 (0.32) a
H_2O	5.95 (0.38) a	2.95 (0.28) b
Enzyme	3.62 (0.40) b	2.90 (0.30) b

*Values in columns followed by different letters are significantly different ($P \leq 0.05$).

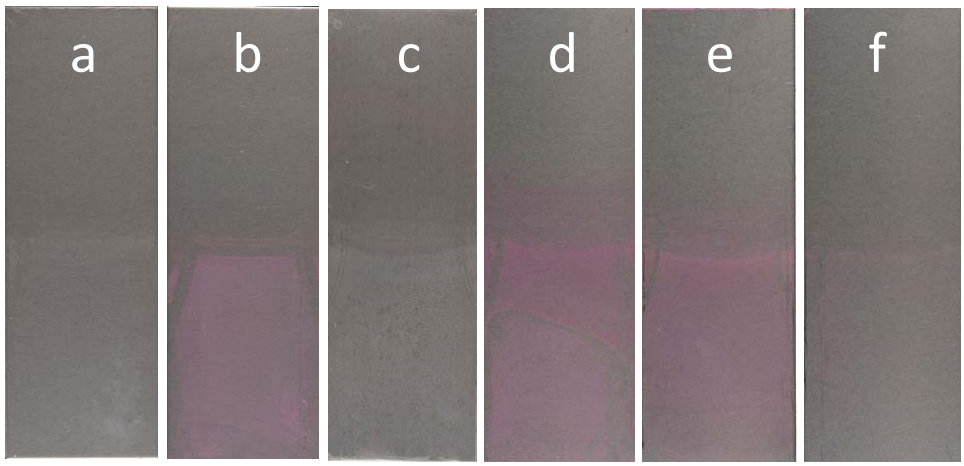

FIGURE 1. Color photographs of selected biofilm-contaminated stainless steel coupons subjected to various species/treatment/erythrosin B dye conditions: (a) *Pseudomonas putida*/NT/no dye; (b) *Pseudomonas putida*/NT/dyed; (c) *Listeria innocua*/NT/no dye; (d) *Listeria innocua* /NT/dyed; (e) *Listeria innocua* /PAA/dyed; (f) *Listeria innocua* /enzyme/dyed.

of a general linear model. All pairwise multiple comparison procedures were performed using the Holm-Sidak method; significance was assigned at $P < 0.05$.

RESULTS AND DISCUSSION

Plate counts of swabbed surfaces of treatment coupons indicated that of the eight treatments, only no treatment, water treatment, and enzyme treatment resulted in detectable levels of viable bacteria in the case of both *L. innocua* and *P. putida* Table 2. In the case of *L. innocua*, water did not significantly remove cells relative to non-treatment, while the enzyme treatment resulted in a 2.5 log CFU/cm² reduction. In the case of *P. putida*, cell counts were reduced by the water and enzyme treatments, which both resulted in a 1.0 log CFU/cm² reduction in viable cells. While the remaining treatments produced no detectable recovery of either *L. innocua* or *P. putida*, dyeing and subsequent visual inspection of the coupons indicated that none of the treatments led to complete removal of the biofilm components. Depiction of this visual inspection was accomplished by photographing each dyed coupon. The resulting photographs of selected treatment coupons, as shown in Fig. 1, provide representative visual images of untreated *L. innocua* and *P. putida* biofilms before and after dying as well as the effect upon visualization of *L. innocua* biofilm of two different sanitizer/cleaner treatments.

Comparison of Figures 1ab and cd show the visual enhancement attained by dyeing both untreated *P. putida* and *L. innocua* (b and d, respectively) compared with undyed coupons (a and c). The red dye contrasted more distinctly with the background than was seen in the undyed coupons. Treatment of *L. innocua* biofilm with PAA had little effect in removing

TABLE 3. Mean color differences as ΔE*, with standard deviation in parentheses, relative to background for stainless-steel coupons contaminated with *Listeria innocua* or *Pseudomonas putida* biofilm, subjected to 8 different cleaner/sanitizer treatments (see Table 1) and subsequently dyed, unless otherwise indicated, using erythrosine B (EB) or crystal violet (CV). Pairwise multiple comparisons by Holm-Sidak method* (n = 3)

Treatment	*Listeria innocua* (EB)	*Listeria innocua* (CV)	*Pseudomonas putida* (EB)
NT, no dye	3.43 (0.65) d	3.43 (0.65) d	3.05 (0.67) c
NT	13.22 (0.25) a	9.50 (0.45) a	11.50 (0.85) a
H_2O	6.85 (0.55) c	6.10 (0.55) b	2.10 (0.90) c
Enzyme	2.89 (0.22) d	1.13 (0.11) e	2.20 (0.50) c
pH = 13	6.50 (0.50) c	4.85 (0.40) c	1.25 (0.75) c
CS1	11.20 (0.55) b	9.75 (0.50) a	1.80 (0.30) c
CS2	6.76 (0.33) c	5.80 (0.30) b	2.40 (0.30) c
PAA	12.63 (0.38) a	8.70 (0.42) a	5.76 (0.22) b
HP	13.28 (0.15) a	9.55 (0.40) a	1.93 (0.60) c

*Values in columns followed by different letters are significantly different ($P \leq 0.05$).

the biofilm components *Fig. 1e*. The enzyme treatment resulted in better removal of the biofilm when *Fig. 1* is compared with *Fig. 1d*. Although visual assessment for the presence of biofilm is apparently enhanced by use of the dye, the method remains relatively subjective, and some means to provide more objective assessment is needed. One method that provides this capability is the CIELAB color difference formula *(10)*. For the present study, quantification of biofilm perceptibility was performed by comparing averaged L*a*b* color values from the stainless-steel background with the treated and dyed portion of the coupons, using Equation 1. The resultant color differences are presented graphically in *Table 3* for *Listeria innocua* untreated and treated biofilms dyed with either erythrosin B or crystal violet and for *Pseudomonas putida* untreated and treated biofilms dyed with erythrosin B. As indicated previously, the perceptible difference in the case of untreated *Listeria innocua* and *Pseudomonas putida* is much greater in coupons dyed with erythrosin B than in undyed coupons. With the exception of PAA, all other treatments produced results similar to water treatment in the case of *Pseudomonas putida*, with remaining biofilm components being nearly imperceptible. In the case of *Listeria innocua*, only enzyme treatment appeared to remove more biomass than water with a mild detergent. Even the commercial cleaner/sanitizers CS1 and CS2 left considerable biomass. Comparison of results of dyeing treated *Listeria innocua* biofilms with crystal violet and erythrosin B showed similar trends in biomass staining of the treated coupons, indicating that erythrosin B can provide a viable alternative to the widely used crystal violet methodology.

Although visual assessment of erythrosin B dyed biofilm on stainless-steel coupons provides an indication of the presence of biomass, it is limited by its inability to provide perceptibility below a value of ΔE*=1–2, even though biofilm components may be present, albeit in low amounts. A further benefit of erythrosin B is that in addition to its color visibility, it is fluorescent, with an emission maximum at ~550 nm, and although the fluorescence cannot be observed visually, it can be observed through use of a fluorescence microscope. Selected images using fluorescence at 100× magnification can be seen in *Fig. 2*. Biofilm components of both *Pseudomonas putida* and *Listeria innocua* with no treatment, followed by dyeing with erythrosin B, can be easily observed in *Fig. 2a and 2b*, respectively. *Figure 2c* shows a captured image of *Listeria innocua* following enzyme treatment. Here, too, biofilm components are clearly observed, whereas under visual observation *Fig. 2f*, the presence of biofilm is barely perceptible. The amount of light emitted by the dyed samples can be quantified by microspectrophotometry. Fluorescence spectra were collected from the images in *Fig. 2* and are presented in *Fig. 3*. Results suggest that quantification of biofilm removal by microscopic fluorescence detection of erythrosin B is more sensitive than color visualization,

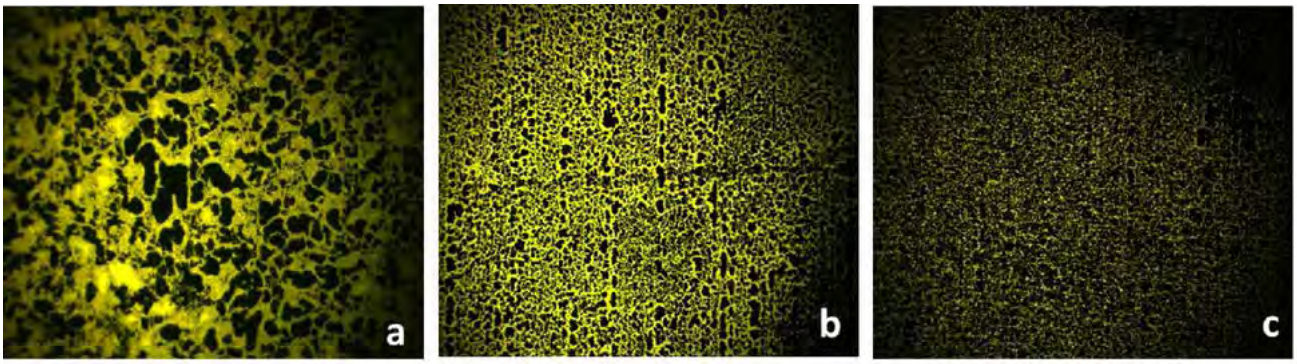

FIGURE 2. Microscopic fluorescent images, at 100× magnification, of stainless-steel coupons contaminated with biofilm, subjected to species/treatment conditions, and subsequently dyed using erythrosin B. (a) *Pseudomonas putida*/NT; (b) *Listeria innocua*/NT; (c) *Listeria innocua* /Enzyme.

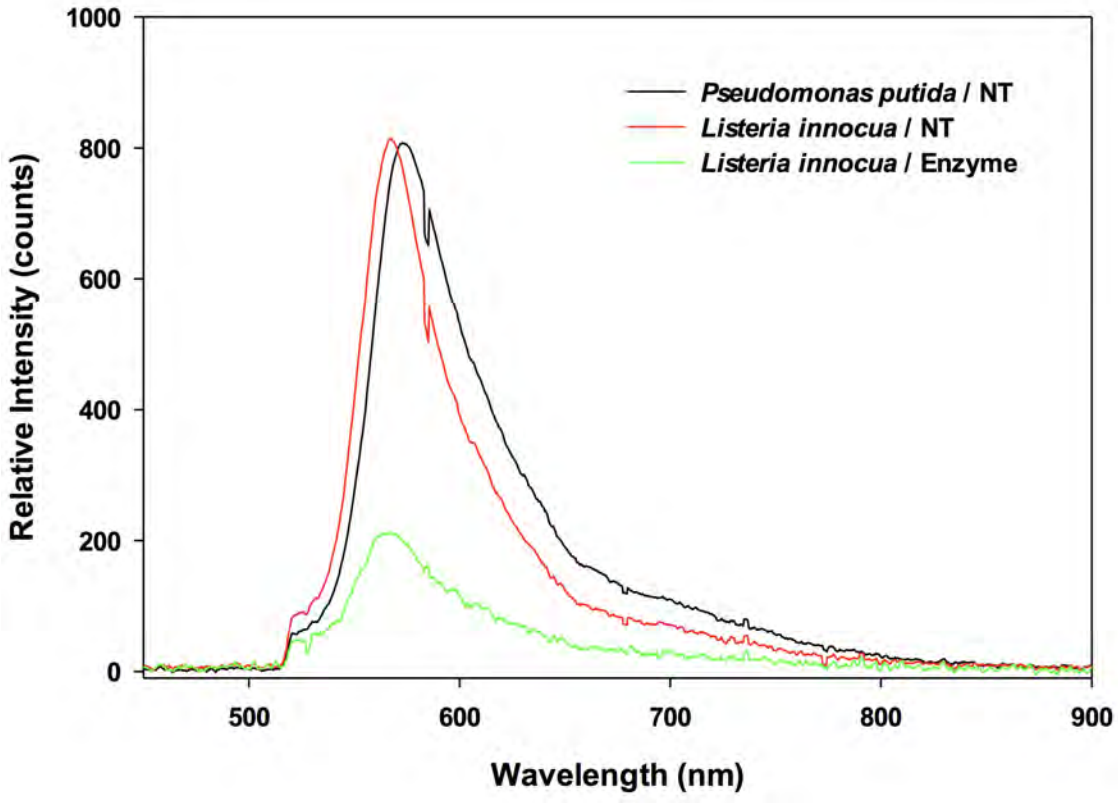

FIGURE 3. Fluorescence spectra of stainless-steel coupons contaminated with biofilm, subjected to species/treatment conditions, and subsequently dyed using erythrosin B.

although on a scale that is currently impractical as a method of assessing hygiene in an industrial setting. While not practical as a rapid or cost-effective method for visualizing the effectiveness of biofilm removal, the fluorescence characteristics of erythrosin B can provide added information to complement visual studies in a laboratory setting.

CONCLUSIONS

Erythrosin B, is a food grade dye that is effective in enhancing the visualization of biofilm components, is useful in laboratory studies evaluating the effectiveness of cleaner/sanitizer treatments in removing biofilm and other contaminants and has the potential to be developed

into a visual aid for detecting contamination in food processing environments during routine inspections. The method is rapid, cost effective, capable of being used on large surface areas that may not be adequately sampled by complementary methods such as swabbing, and potentially useful in increasing the efficiency of swabbing regimes by allowing visual enhancement of suspect areas. The present study utilized *Listeria innocua* and *Pseudomonas putida* as biofilm surrogates to obtain promising results, but future studies are being planned to test the dye methodology on additional mono-species as well as bi-species and environmentally obtained biofilms to ensure that a broad spectrum of potential biofilms can be visualized. In addition, the method will be investigated for its utility in visualizing biofilms on other processing surfaces, including Teflon, glass, rubber and polyurethane.

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