The detection of *Escherichia coli* biofilm in green oak with spectroscopic imaging techniques based on confocal laser scanning microscope

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Abstract. Microbial recontamination in post-harvest products, specifically for fresh cut fruits and vegetables, often raises concern of consumer health safety since pathogen bacteria readily forms sticky biofilm for their protection that often render ineffective of cleaning or disinfection process. Therefore, tracking biofilm rather than bacteria cells may also be used for indication of microbial recontamination. This research focuses on acquisition of spectroscopic imaging and information for *E. coli* biofilm adherent on green oak leaf at microscopic level for visible bandwidth. A confocal laser scanning microscope (CLSM) was applied to investigate for the evident of biofilm formation. In order to enhance spectroscopic signal, staining surface sample with propidium iodide, commonly used for staining dead cell, was specifically investigated to a potential use for determination of *E. coli's* biofilm as an evident of recontamination occurrence. Green oak leaf samples were inoculated with a small drop of viable *E. coli* of $2.7x10^9$ CFU/ml covering around 7x7 mm^2 and all samples were spectroscopic imaged every day for 7 days. Absorbance spectrum acquired by CLSM for the *E. coli* inoculated samples presented three identity peaks at wavelengths of 620, 670, and 690 nm but the latter identity wavelength appeared to include common autofluorescence of leaf surface physiology so arguable interpretation is unavoidable for 690 nm. In opposite, the identity peaks at 620 and 670 nm presented strong detection of *E. coli* 's biofilm for storage time beyond day 5 at a significant level of confident 99%. However, the lowest minimum detection limit in term of *E. coli* concentration required more precisely experimental validation.

Keyword: *Escherichia coli*, Biofilm, CLSM, Spectroscopic

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1 Introduction

Biofilm is a matrix of exopolysaccharides, protein, DNA and some of lipid produced by bacterial cells that attached on the surface of various substrates and may be called as extracellular polymeric substance (EPS) [1]. The bacterial cells with biofilm have high resistance to disinfectant, then consumption of cross contamination in food products can be harmful to consumers. Consequently, biofilm detection methods was indeed useful for food safety and quality inspection.

Since 1990, there have been several studies about biofilm formation and microbial growing rate using spectroscopic technique by determining the strains absorbance value with crystal violet staining [2]. Subsequently, the technique of biofilm examination was developed by combining spectroscopic and imaging. Brider *et al*. [3] used the Syto9 for DNA stain and live/dead cell stains including analysis responsibility with a confocal laser scanning microscope (CLSM) in 2010. CLSM analysis becomes a popular tool for study of microorganism and biofilm. By virtue of the staining reagents, CLSM can provide both image and specific reflective spectra correspondent with absorbance wavelength. Therefore, many researchers have been characterize biofilm produced by various bacteria based on their specific spectrum. Kim et al. [4] reported that the biofilm formation of *E. coli* occurred on several types of surface materials, namely HDPE, Formica-type plastic laminate , Granite uniform black color, granite non-uniform, and stainless steel and its major spectrum was characterized at wavelength of 470 nm. Rodney 2002 [1] reported that dapi maker was a good staining reagent for biofilm with a specific wavelength of 450 nm.. In 2017, Chow goon Ng *et al* [6] used antibody markers, Alexa Fluor-tagged goat and anti-rabbit antibodies and scanning electron microscope to investigate the biofilm formation on vegetables. The vegetables without cultures always have the response of autofluorescence at green and red colors (550–700 nm approximately) while biofilm formation on the vegetables with culture responded to specific wavelength dependent on the antibody markers.

The purposes of this research was to reinvestigate whether propidium iodide can be used to characterize biofilm formed by E. coli on the surface of green oak salad. A confocal laser scanning microscope (CLSM) was applied to identify the identity of absorbance wavelength responsible for E. coli's biofilm stained by propidium iodide. Spectroscopic imaging technique was proposed for risk evaluation of green oak salad consumption.

2 Materials and methods

The culture growing reagent such as Nutrient Agar,,Trypic soy Broth was purchased form Sigma Aldrich .For the 1XPBS and Propidium Iodide staining reagent was purchased from Invitrogen Molecular Probes(UK).

2.1 Samples preparation

The green oak was purchased from Suranaree University of Technology farm, Thailand. Samples were washed with DI water and added 1000 ml of 100 ppm latron CS7-100 for 30 min for decreasing a surface tension on green oak leaves. The samples were washed with DI water and dried at ambient conditions.

2.1.1 Biofilm formation on green oak

Escherichia Coli TISTR371 was received form Biodiversity Research Center, Institute of Scientific, Thailand. It was first plated on nutrient agar (Sigma Aldrich) and incubated at 37ºC for 24 hrs. Single colonies were transferred to 10 ml of Trypic soy broth then incubated at 37ºC for 24 hrs. After 24 hrs, 10 µl of culture was dropped on green oak leaves at each position then incubated at 8ºC for 5 days. The sample was observed inspected at 0, 1, 3, 5 and 7 days, respectively.

2.1.2 Confocal laser scanning microscope (CLSM)

The samples with biofilm formation process were rinsed with DI water for removing non-adherent bacteria. Then, 50 µl of propidium iodide (PI) staining was dropped on the leaves and incubated in dark at room temperature for 30 minutes. The sample were washed with DI sterile for cleaning non stained reagent out of surface. The leaves were cut and placed on glass slide. Then, they were analyzed by using Nikon 80E inverted Confocal Laser Scanning Microscope in confocal mode and spectral mode with laser excitation/emission at 488/500-550 nm and 561/570-620 nm.

2.1.3 Scanning electron microscopy (SEM)

The sample preparation process for SEM was shown in Figure 1. The freeze vegetable and culture cell sample with different conditions were cut to 1x1 cm^2 and soaked in 2.5% of glutaraldehyde in 0.5xPBS (pH 7.2). Then, the samples were washed 3 times with 0.5xPBS. After washing process, the samples were incubated in 1% osmium tetroxide for 2 hrs and washed with sterile DI water 3 times. The samples were dehydrated with acetone sequentially (20%, 40%, 60%, 80%, and 100% of acetone). After dehydration process, samples were dried by critical point dryer. The morphology of biofilm and bacteria cells were analysed by FIB-SEM Carl Zeiss Model Auriga.

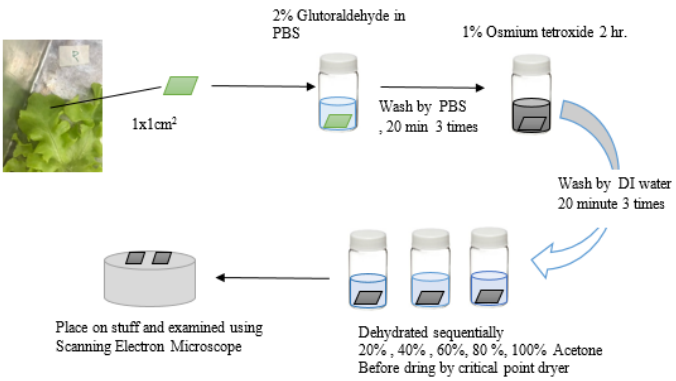

Fig. 1. The sample preparation process for Scanning Electron Microscope.

3 Results and discussion

3.1 Confocal laser scanning microscope

Evident of *E. coli* and its' biofilm formation on the leaf surface of green oak salad was characterized by using propidium iodide staining technique and CLSM imaging under a confocal mode at 488 and 561 nm excitation. Selective green and red color channels of CLSM images of no culture area without PI staining (Figure 2A), no culture area with PI staining (Figure 2B), and *E coli* doped-area with PI staining (Figure 2C) showed that stomata of control samples regardless of PI staining clearly appeared in green, but little or no emission in red as shown in Figure 2A and 2B [6]. However, a selective wavelength (690 nm) image of no culture area with PI staining showed dominant red color, but no red image was noticeable at the other two red wavelengths of 620 and 670 nm as seen in the case of *E coli* doped-area with PI-staining (Figure 3). The spectral signal at 680-690 nm represented the chloroplast with chlorophyll A. The Chlorophyll A was commonly existed in green plants and emitted at wavelength 688 and 730 nm after being excited by blue-green fluorescence [7-9]. Therefore, the spectral wavelength of 620 and 670 nm can be used as unambiguous indicators for either *E. coli* or its' biofilm.

Since biofilm produced from *E. coli* doped on the green oak leaf is unable to respond to fluorescence excitation, propidium iodide staining reagent was used to enhance the biofilm culture intensity in this case. Propidium iodide is the DNA staining reagent which responds to 535 nm excitation with 617 nm emission. Figure 2C showed that the leaf with culture staining has high intensity than control culture. The rod cell of *E. coli* was dispersedly found around this area. The red color fluorescence of green oak indicated the emission of propidium iodide staining.

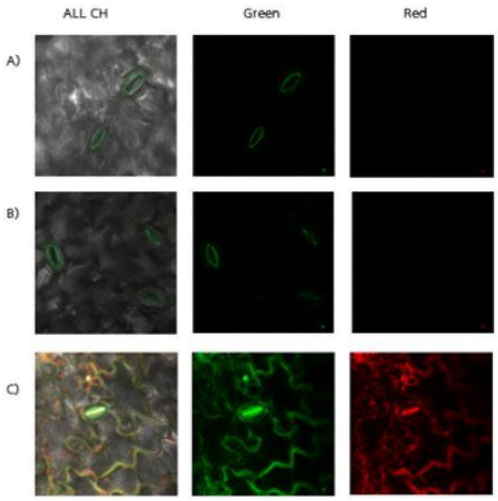

Fig. 2. CLSM images of green oak leaf samples with a magnificent of 40x **A)** No-culture area without PI staining **B)** No-culture area with PI staining **C)** *E coli* doped-area with PI staining.

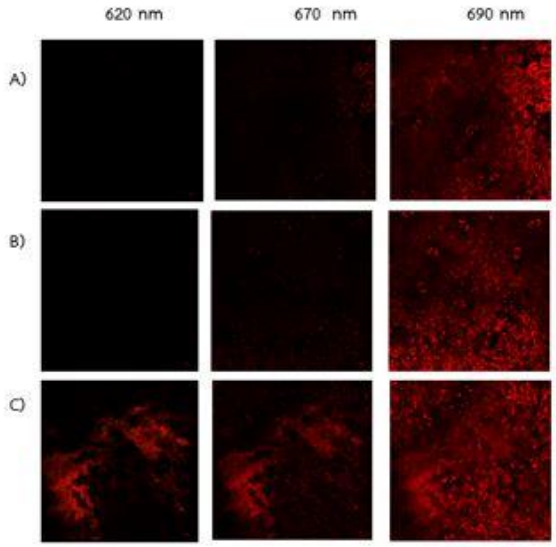

Fig. 3. Three selective wavelengths of CLSM images of green oak leaf samples with a magnificent of **40x A)** No-culture area without PI staining **B)** No-culture area with PI staining **C)** *E coli* doped-area with PI staining.

The spectral of red color from plant auto fluorescence and PI emission were compared in CLSM spectral mode (Figure 3). All of the samples' response was presented at range 670-690 nm and range 680-690 nm similar to the report of Krause *et al.* and Kodama [7-8]. On the other hand, A sample of culture stained would respond at a strong wavelength of 620-630 nm correlated with the culture with PI straining which was presented the fluorescence emission at 617 nm and the wavelength may shift for about ±3 nm.

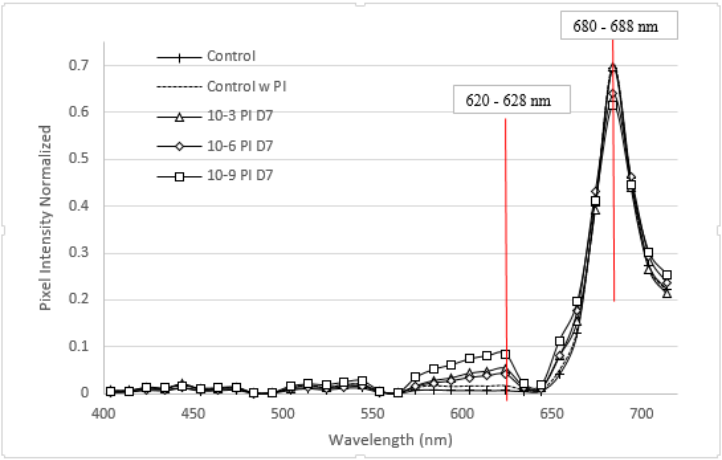

Fig. 4. The normalized spectral profiles of green oak salad for control sample without PI staining, control sample with PI staining and culture dilution with PI staining.

In Figure 4 , the existence of plant autofluorescence and biofilm were substantiated by PI staining. The autofluorescence of vegetables commonly found around 680-690 nm. The results presented that the autofluorescence between green oaks with and without culture was indifferentiable.

The autofluorescence signal of stomata area was stronger than another area related to chlorophyll content. Nayara in 2013 [9] reported the leaves area with high stomatal index and stomatal density (stomata/mm^2) will induce the high chlorophyll .Morever ,chlorophyll level depends on the stomatal conductance. Sami et al [10] describe the effect of stomata on chlorophyll content in the leaf. The area with high stomatal index but low stomatal conductance will make the low chlorophyll content. Therefore, the signal intensity of autofluorescence at 670-680 nm might not equal depends on analytical areas with stomata index and stomatal conductance.

The biofilm classification of samples were indicated in the spectrum. The pixel intensity was normalized and analysed by T-test analysis and the results presented in Table 1. The pixel intensity at 620 nm is different from at day 5 to day 7 at significant 0.1. On the other hand, control, control with PI staining at 620 nm, and all samples at 680 nm are not different at 90%. This technique has advantages that it is non-destructive test, provide a large area, and take a shorter time than the convention method (Table 2.). However, the limitation of this method cannot detect the biofilm from day 1 to day 3 because the biofilm formation is low concentration.

Table 1. Normalized spectral intensity to classified Green oak with *E. coli* biofilms.

Sample	620 nm		680 nm	
	Day 5	Day 7	Day 5	Day 7
Control without PI	0.009 ± 0.003^{ab}	0.007 ± 0.02^{ab}	0.610 ± 0.06^{ab}	0.696 ± 0.016^{ab}
Control with PI	0.011 ± 0.005^{ab}	0.017 ± 0.01^{ab}	0.552 ± 0.04^{ab}	0.703 ± 0.025^{ab}
10^{-6} PI	0.023 ± 0.01^{aa}	0.042 ± 0.01^{aa}	0.601 ± 0.06^{ab}	0.639 ± 0.033^{ab}
10^{-9} PI	0.023 ± 0.08^{aa}	0.048 ± 0.004^{aa}	0.671 ± 0.03^{ab}	0.616 ± 0.060^{ab}

aa= has different at significant 0.1.

ab = has not different at significant 0.1.

*Biofilm formation on green oak at low concentration (day 0- day 3) has no different with control samples.

Table 2. Comparison of biofilm detection between convention methods and spectroscopic techniques.

Methods	Sample charecterization	Testing time	references
Tissure culture plate (TCP)	Size reduction by stomacher , dilution and staind by Crystal violet	24 hr	Pragyan et al [11]
Tube method (TM)	Size reduction by stomacher , dilution and staind by Crystal violet	24 hr	
Congo Red Agar (CRA)	Size reduction by stomacher , dilution	24 - 48 hr	
Spectroscopic techniques	Surface	1 hr up (depend on concentrations) *	

* Biofilm studies on vegetables with conventional methods was incubated for 24 hr. While, the testing time of the spectroscopic method depends on biofilm concentration. The high concentrations of biofilm on vegetables can be detected at that time. The low concentration of biofilm must be incubated for enhanced the signal.

3.2 *E. coli* biofilm formation by scanning electron microscope

The morphology and structure of biofilm formation of *E. coli* was analysed by SEM (Figure 5). The result showed that biofilm density has been increased with the times. First state, the single cell of bacteria used flagellum for cohesion the vegetable surface (Figure 5B). Second stage, figure 5C showed the cells of bacteria divided and the *E. coli* cells created the EPS structure for the coherent of cells-surface and cell-cells. Final state, the bacteria cells in biofilm structure would be grown and maturated (Figure 5D and 5E) [12].

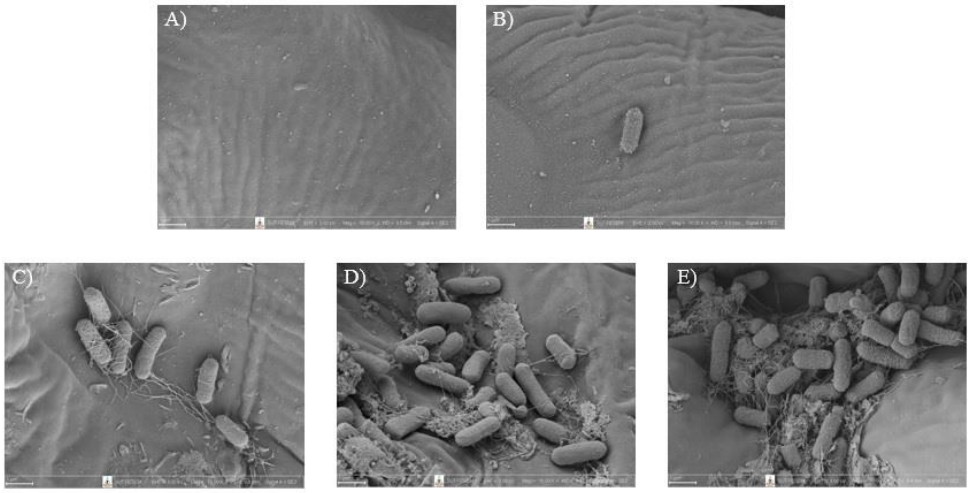

Fig. 5. *E. coli* biofilm formation on green oak salad by SEM. 10K Magnification A) control, B) After Incubated 30 minutes, C) Biofilm formation after incubated 8°C 24 hrs, D) 72 hrs, and E) 120 hrs.

The comparison results of SEM and CLSM of biofilm formation found that the autofluorescence of the green oak leaves was confirmed in 120x of CLSM and 1000 of SEM at day 5. The most of cultures were attached to the leaves rather than stomata. Therefore, the response of red autofluorescence at 680 nm was the stomata area and the response of red autofluorescence at 620 nm was leaves. The result showed the concentration of autofluorescence at stomata area was relatively higher than at leaves.

4 Conclusions

The purpose of research has been applied spectral signal for detecting *E. coli* biofilm area on green oak salad. Green oak has a mainly responsive signal at 620 and 680 nm. The contamination area was classified with a staining reagent emission spectrum. For this case, it is propidium iodide at 620 nm emission and the response of autofluorescence response in plant was reported to 680 nm. The significance of signal intensity was not less than 90% for sample classification with and without *E. coli* biofilm. The results found that culture growing rate at storage condition 8°C has been decreasing when time has been increasing, but biofilm intensity directs variation with times.

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References

1. M.R. Donlan, Emerging Inflections Diseases **8**, 881-890 (2002).
2. O'Toole, A George, Journal of Visualized Experiments, **47**(2011).
3. A.Bridier, F.D. Brissonnet, R. Briandet, Journal of microbiological methods volume **82**, 64-70 (2010)
4. W.J. Kim, M.S. Kim, B.K Cho, Journal of Food Engineering **99**, 314-322 (2010)
5. Chow goon Ng., Mun Fai Loke, Food Microbiology **62**, 68-76 (2017)
6. G.H. Krause, E. Weis, Annu.Rev. Plant physiol. Plant Mol Biol **42**, 313-349 (1991)
7. K. Kodama, Plos one. 2016
8. C. D. Everard, M.S. Kim, H.Y Lee. Journal of Food Engineering **143**, 139 – 145 (2014)
9. N.C. de Melo, L.A Beijo, Amazonnian Journal of Agricultural and Environment Sciences **56**, 80-88 (2013)
10. Sami Ullah Qadir, Vaseem Raja, Weqar A. Siddiqui. Ecotoxicology and Environmental Safety **129**, 320-328 (2016)
11. S.P Pragyan, U. Chaudhary, S.K. Dube, Indian Journal of pathogen&microbiology **59(2)**, 177-179 (2016).
12. S. Liu, C. Gunawan, N. Barraud, Environmental Science & Technology **50**, 8954-8975 (2016.)