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Abstract. Salmonella is pathogenic bacteria that caused foodborne diseases which being called Salmonellosis. Prevalence of Salmonellosis that being caused by *Salmonella thypimurium* in Indonesia is quite high. However, detection of Salmonella bacteria in food still limited, complicated, and required a lot time. Sensitive optical assay for *Salmonella thypimurium* paper based detection has been developed by integrating sandwich assay between antibody-antigen complex and alkaline phosphatase enzyme that produce visible bluish-purple colour with presence of NBT-BCIP substrate. The results showed that Limit of Quantitation of detection is 105 CFU mL^{-1} with detection time 15 minutes. Linearity test between Colour intensity that produced from Salmonella concentration presence on samples showed that detection has good linearity. Selectivity test exhibited excellent sensitivity with good discrimination against *Escherichia coli*.

1. Introduction

Food safety is an important factor in food requirement in order to avoid the occurrence of disease. A disease that occurred through food consumption referred as food poisoning or food-borne disease [1]. Food-borne disease is commonly caused by the presence of pathogenic microorganism such as Salmonella [2,3,4]. Salmonella does not necessarily cause change in color, smell, or taste of foods, but if there's a lot of Salmonella presence in the foods, then Salmonella will cause symptoms which is called Salmonellosis. The cases of Salmonellosis are still a major problems in food safety worldwide as reported by Pui *et al* [5,6] that there are 16 million cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths due to Salmonella. The risk of Salmonellosis is increased 5-10% each year [7,8]. Prevalence of Salmonellosis caused by *Salmonella thypimurium* in Indonesia was 1.6% in 2007 with highest cases were found in school age children [9]. However, Indonesia still use conventional methods which are limited, complicated, and needed approximately 5-7 days to complete the analysis to determine *Salmonella thypimurium* presence in foods[10,11,12]. This condition made Salmonellosis became more complicated as we hardly can detect the presence of *Salmonella*

thypimurium in our food. In order to solve that problem, immunosensor based Biosensor can be an alternative of a rapid, practical and accurate *Salmonella thypimurium* detection.

Salmonella thypimurium detection is fabricated using colorimetric biosensor principle. The test result can be observed directly due to color change as indicator of positive testing result, while negative testing result will not develop any color change [13,14]. In this paper, a paper based detection and sensitive optical assay for *Salmonella thypimurium* has been developed by integrating sandwich assay between antibody-antigen complex and alkaline phosphatase enzyme that produce visible bluish-purple color with presence of NBT-BCIP substrate. This immunosensor developed are expected to be an alternative method for Salmonella detection.

The aim of this research is to developed sandwich type-optical immunosensor which is can be an alternative of Salmonella detection in Indonesia. The relevant experimental variables, including the LoQ (Limit of Quantitation), detection time, linearity and selectivity is examined and optimized both qualitative and quantitative.

2. Materials and Methods

2.1. Bacteria and Media

Salmonella choleraesuis serotype *typhimurium (S. Typhimurium)* and *Escherichia coli* was used for the experiments (obtained from Food Microbiology Laboratory, Faculty of Agriculture Technology, Brawijaya University) Indonesia. Culture of *Salmonella typhimurium* were prepared by incubating the cultures in Natrium Agar (NA) slant at 37˚C for 24 h and culture of *Escherichia coli* were prepared by incubating the cultures in Lactose Broth (LB) at 37˚C for 24 h.

2.2 Reagent and Antibody

Salmonella typhimurium polyclonal antibody was purchased from Bioss. Purified rabbit anti-Salmonella igG-AP (Chemicon), BSA (Nacalai), Gluteraldehyde (Sigma), and Tween 20 (Nacalai) were purchased from Bioscience Laboratory, Brawijaya University. Buffer Saline Phospate (Merck) and NBT-BCIP (Thermo Scientific) were purchased from Biomedical Laboratory, Brawijaya University.

2.3 Preparation Immobilization of Antibody

Preparation steps are done by sterilizing the Whatman paper #1 that is used as platform (110˚C, 60 minutes). In spite of that, reagents required also been made, such as 1% PBST (15µL Tween 20 in 148 µL Buffer Phosphate Saline/BPS), 0.1% BSA (0.1 mg BSA in 100µL PBST), 5% Gluteraldehyde e (10µL Gluteraldehyde in 190µL PBST) and secondary antibody (combination of *Salmonella typhimurium* antibody and anti-rabbit AP-labelled antibody) as well as 0.1 mg/ml primary antibody (20 µL 1 mg/ml *Salmonella typhimurium* antibody in 180µL BPS).

2.4 Fabrication of Biosensor

Sandwich type-optical immunosensor is fabricated using modified Zourob[15]and Cao[16]steps of fabrication. Fabrication was done by the following steps: 4µl secondary antibody is dripped onto the reaction zone and left it until dried. Then, 4µL Gluteraldehyde is dropped to result zone and left to stand until it's completely dry prior to 4µl primary antibody which is added onto the result zone. After the paper is completely dry, the result zone is washed using PBST to discard unimmobilized primary antibody. After washing step, 4µL of NBT-BCIP substrate is added to the result zone and left to stand until it's dry. Lastly, 4µL BSA is added as blocking agent to block areas which did not bind to any antibody.

Figure 1 shows the fabrication steps. The detector is then incubated for 4˚C, 24 hours before it is ready to use.

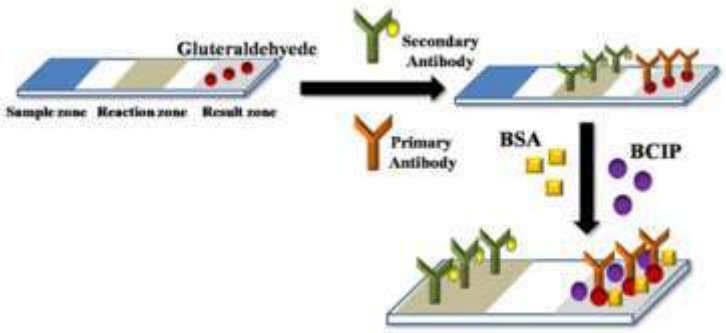

Figure 1. Schematic representation of sandwich type-optical immunosensor based *alkaline phosphatase* enzyme construction

2.5 Experiment

The experimental was conducted to determine Limit of Quantification (LoQ), linearity, detection time, selectivity and storage stability of the detector. Limit of Quantitation of the immunosensor is determined by modification of method published by Aloraefy[17]. Six different Salmonella concentrations (0, 10^4; 10^5; 10^6 ; 10^7 ; and 10^8 CFU mL^{-1}) are used as samples and the result of the tests is analyzed using Hex Color Finder to obtain RGB value of each concentrations. Then, RGB-*Salmonella* concentration standard curve is made using Ms. Excel. Limit of Quantitation (LoQ) is calculated from equation line of standard curve with the value of Y-axis is the RGB value of control/ background (0 CFU mL^{-1}). Linearity of sandwich type-optical immunosensor is determined by R2 value of RGB-Salmonella Concentration standard curve of LoQ variable test.

Detection time is determined by measure the time duration when sample is dropped into sample zone until color is developed and stable at result zone, indicated that alkaline phosphatase enzyme react with NBT-BCIP substrate to produce blue/violet colored products.

Selectivity is determined using modified method from Bhalla[18].The detectors were tested using control (BPS); *Salmonella typhimurium* and *E.coli* culture. Last experimental variable is storage stability which was determined by modified method from Rathee[19]. Three fabricated immunosensors that had been made at the same time were tested using *Salmonella typhimurium* culture at different time. The platform stability was tested for 0, 2 and 4 weeks after incubation time.

2.6 Apparatus

The test results was documented using scanner (Canon MP250 Series) and analyzed using Hex Color Finder in order to obtain the RGB value of each test results. Hex Color Finder (HFC) is an application for Windows which created by NZ Works and help users to get RGB colors to detected hexadecimal value (e.g. #2E505C). Then, the data acquired was analyzed to determine overall performances of the detector, both quantitative and qualitative.

3. Results and Discussion

3.1 Limit of Quantitation (LoQ)

Limit of Quantitation is the lowest analyte concentration that still can be detected [20]. RGB value that obtained from each concentration is 135.33(0 CFU mL^{-1}),137(10^4 CFU mL^{-1}), 135.67 (10^5 CFU mL^{-1}), 134.67 (10^6 CFU mL^{-1}), 130 (10^7 CFU mL^{-1}), and 97 (10^8 CFU mL^{-1}), after obtained these RGB value, Standard Curve RGB-Concentration is being made (Figure 2). The LoQ value of the immunosensor is 10^5 CFU mL^{-1} , this value is obtained from calculating X-axis value from Standard curve RGB equation. According to NCDC[1] and Yousef and Carlstrom[21] infection dose of *Salmonella* that can caused food poisoning is varied up to 10^9 CFU mL^{-1}. Based on NCDC [1], the immunosensor that can be developed in this paper can detect the presence of *Salmonella typhimurium* at lower concentration (10^5 CFU mL^{-1}) than the maximum concentration (10^8 CFU mL^{-1}) that can

caused food poisoning. Therefore this immosensor performances is good for *Salmonella typhimurium* detection. The color that being developed from each concentration test (Figure 3).

Linearity is linear change from measurement results because of analytes (presence in a certain) concentration range [18] Standard curve had R^2value 0.642 which can be interpreted that the linearity of the standard curve still quite good. That results mean RGB values that being obtain from each tests is quite proportional to tested *Salmonella* concentrations.

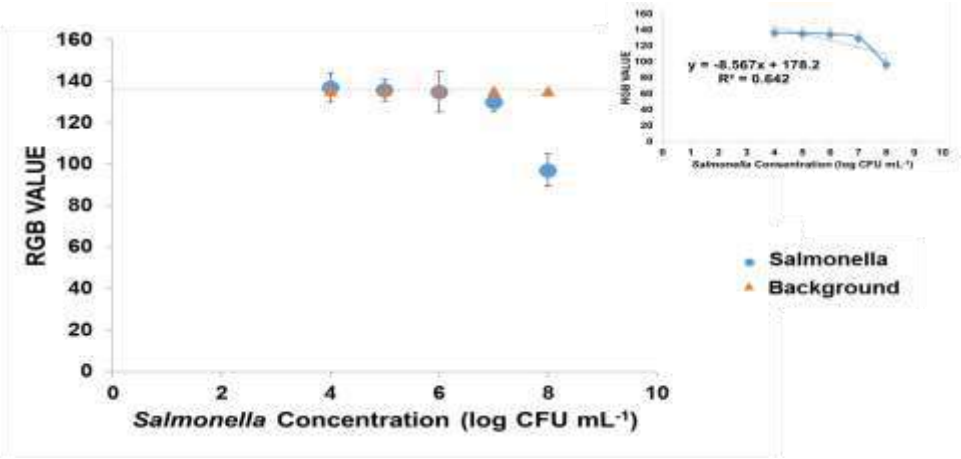

Figure 2. Calibration of *Salmonela thypimurium* concentration by sandwich hybridization using Hex Color Finder (HFC)

A **B** **C** **D** **E** **F**

Figure 3. Color developed from different tested *Salmonella thypimurium* concentrations (A) 0, (B) 10^4, (C)10^5, (D)10^6, (E)10^7 and 10^8 log CFU mL^{-1}

3.2 Detection Time

Detection time analysis of the immunosensor resulted the visible and stable colour change occurs after 15 minutes of sample addition (Figure 4). Conventional detection method of Salmonella required more than 5 days for complete isolation and confirmation processes [10,11, 12]. Therefore, immunosensor that was successfully developed in this paper has less detection time compared to conventional detection method of Salmonella.

A **B** **C** **D**

Figure 4. Color developed from 10^5log CFU mL^{-1} *Salmonella* concentrations with different time incubation (A) 0, (B) 5, (C) 10 and (D) 15 minute.

3.3 Selectivity

Selectivity is the ability of a molecule to discriminate interaction partners. A selective binder shows little cross-reactivity[22] means that it recognizes a given partner with much higher affinity than other partners. Selectivity test results showed that the detector can only developing color when *Salmonella* is present, indicated by RGB value obtained is lower than the control while *E.coli* testing

result showed that there was no significant differences with the control (Figure 5).

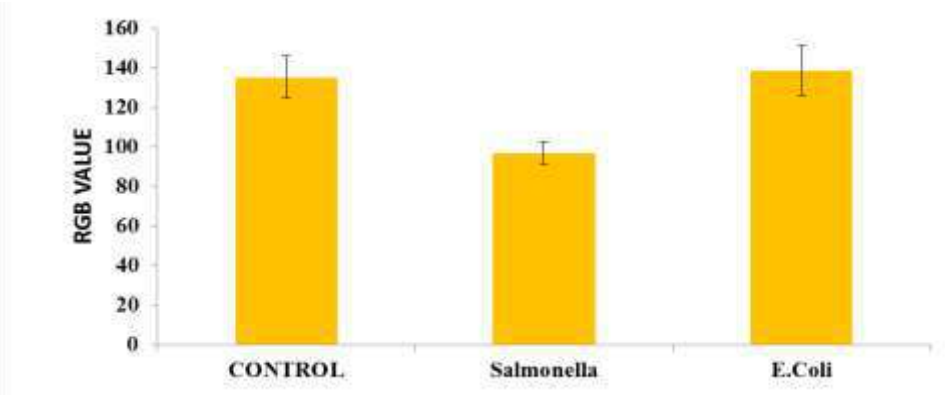

Figure 5. Use of sandwich-type optical immunosensor to measure *E.Coli*, strain BL2(DE3). The response is shown to *E.Coli* ($1x10^5$ CFU mL^{-1}), Salmonella ($1x10^5$ CFU mL^{-1}) and control (without bacteria)

3.4 Storage Stability

RGB values of three immunosensors that had been made and tested in different weeks using Salmonella culture was 116.67 for week 0, 113 for week 2 and 136.67 for week 4. This immunosensor performed good stability for 4 weeks ,indicated that there is no color development at week 4 as can be seen in Figure 6. The addition of stabilizer and encapsulation of enzyme might extend the stability of platform [23].

A **B** **C**

Figure 6. Stability sandwich-type optical immunosensor based on the alkaline phosphatase enzyme (A) 0, (B) 2 and 4 weeks

4. Conclusion

A sandwich-type optical immunosensor that had been developed in this study can be used for selective detection of *Salmonella thypimurium.* The biosensor presented here provides real time detection (15 minutes) with high sensitivity, quite good color linearity and excellent selectivity against *E.coli*. This result has shown promising application in the future as alternative *Salmonella thypimurium* detection method. However, further research about paper types, concentration of antibody and enzyme that being used and also immobilization method of antibody onto the paper should be conducted in order to optimize overall performances of detection.

Acknowledgments

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