

Study Characterization of Aflatoxin-Whole Cell Biosensor based on Co-transformed *Escherichia coli* BL21(DE3) with pKCYP and pSOSGFP

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ABSTRACT

Aflatoxin-B₁ (AFB₁) is one of secondary metabolite which is produced by *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus*. This mycotoxin is commonly found in agricultural products such as corn and beans. Nowadays, the detection method of AFB₁ is still expensive for daily routines; therefore it requires an alternative approach for reducing the cost. Through synthetic biology approach, recombinant *E. coli* is currently used as biosensor for detection of AFB₁. In this study, construction of Aflatoxin-Whole Cell Biosensor was obtained by co-transformation of *Escherichia coli* BL21 (DE3) using plasmid pKCYP and plasmid pSOSGFP. Plasmid pKCYP contains CYP 3A4 which is regulated by constitutive promoter, while pSOSGFP contains Green Fluorescent Protein (GFP) which is regulated by SOS promoter. The oxidation process of AFB₁ by CYP 3A4 becomes AFB₁-8,9-epoxide leads to the damage of DNA. This will activate SOS promoter to produce GFP. The first experiment is to test the response of SOS promoter when DNA damage occur. The damaged can be simulated using UV light exposed to recombinant *E. coli*. pSOSGFP-transformed *Escherichia coli* BL21 (DE3) was exposed by UV light in various range of time; 0 minute, 30 minutes, 60 minutes, and 90 minutes. Result shows that the GFP intensity was increased significantly as longer as the exposure to UV light was performed. Co-transformation of *Escherichia coli* BL21 (DE3) by pKCYP and pSOSGFP using multiple-antibiotic resistant's selection (Chloramphenicol and Kanamycin) showed the colony formation on the LB agar medium. These co-transformed colonies were characterized by standard-curve and growth-curve formulation in order to

determine the exponential phase of recombinant *E.coli*. Based on the growth-curve equation, maximum exponential phase is in the range of OD_{600} 0.896-1.314. During this phase, biosensor was exposed by AFB₁ with the concentration of 2 ppb and 20 ppb in various range of time; 0 minute, 30 minutes, 60 minutes, 90 minutes, and 120 minutes. Biosensors treated by 20 ppb of Aflatoxin-B1 showed higher GFP intensity than the biosensors treated by 2 ppb of Aflatoxin-B1. In addition, biosensors treated by AFB₁ showed lower growth rate in comparison with non-recombinant *E. coli* control.

Keywords: Aflatoxin-B1, CYP 3A4, SOS promoter, Green Fluorescent Protein

INTRODUCTION

Aflatoxin is one of secondary metabolite which is produced by *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus* [1]. Aflatoxin is often found in agricultural commodities such as corn, beans, grains, cereals, and dried fruits, as contaminants [2]. There are several types of aflatoxins found in the nature, but Aflatoxin-B1 (AFB₁) is the most dominant type and the most toxic agents [3]. Based on epidemiological studies, exposure to AFB₁ continuously implicated in liver damage [4].

Nowadays, the detection method of AFB₁ in foodstuffs and agricultural products are various, such as TLC, HPLC, ELISA [5], and fluorometric method [6]. But all of these analytical procedures involve a series of step which require adequate practical ability. Furthermore, this method depends on the presence of laboratory equipments and takes time. In addition, the use of specific chemical reagents and compounds in HPLC and ELISA make these methods less affordable for routine use in developing countries [7].

In this study, through synthetic biology approach, we developed Aflatoxin-Whole Cell Biosensor based on co-transformed *Escherichia coli* BL21(DE3) with pKCYP and pSOSGFP. Plasmid pKCYP contains CYP 3A4 which is regulated by constitutive promoter. It is detected resistance to chloramphenicol. While pSOSGFP contains Green Fluorescent Protein (GFP) which is regulated by SOS promoter and has resistance to kanamycin. AFB₁ epoxidation become AFB₁-exo-8,9-epoxide by pKCYP will cause DNA damage which triggers SOS promoter activation to express GFP [8].

As the first step in developing Aflatoxin-Whole Cell Biosensors, the growth characterization of biosensor and AFB₁ exposure was conducted. Co-transformation of *Escherichia coli* BL21 (DE3) with pKCYP and pSOSGFP using multiple antibiotic selection methods. For further, the growth phases of co-transformed *E. coli* was characterized by the determination of standard curve and growth curve. During the peak of exponential phase, biosensor was exposed to AFB₁ with concentration of 2 ppb and 20 ppb in the period of time 0 minutes; 30 minutes; 60 minutes; 90 minutes; and 120 minutes. GFP expression and effect AFB₁ on the biosensor growth was observed later.

MATERIALS AND METHODS

Confirmation of pKCYP and pSOSGFP

Confirmation of pKCYP and pSOSGFP were conducted with restriction analysis and PCR method. In restriction step, restriction enzyme *EcoRI* and *PstI* were applied for both plasmid. In the PCR step, we used universal primers VF2 (5'-TGCCACCTGACGTCTAAGAA-3') and VR (5'-ATTACCGCC TTTGAGTGAGC-3'). The PCR results were confirmed by the electrophoresis using 1% agarose gel at 100V voltage in 30 minutes.

Co-transformation of *Escherichia coli* BL21(DE3) with pKCYP and pSOSGFP

50 µl of *Escherichia coli* BL21(DE3) competent cell were co-transformed with pKCYP and pSOSGFP plasmid, 5 µl of each. Co-transformation protocol based on the modification of iGEM protocol. As a negative control, used *Escherichia coli* BL21(DE3) without both of plasmid, *Escherichia coli* BL21(DE3) containing pKCYP, and *Escherichia coli* BL21(DE3) containing pSOSGFP. 100 µl of each culture were inoculated in LB agar containing chloramphenicol and kanamycin by spread method. Culture then were incubated during 14 hours in incubator 37°C.

SOS Promoter Test

Escherichia coli BL21(DE3) were transformed with pSOSGFP plasmid by standard iGEM protocol [9]. After 14 hours incubation, a single colony grown on LB agar containing kanamycin was picked and grown as liquid culture, which is incubated 14 hours in shaker incubator 37°C. Culture then was exposed to UV C 15 watt for 0 min (control), 30 minutes, 60 minutes, and 90 minutes. As a negative control, we used *Escherichia coli* BL21(DE3) without pSOSGFP. GFP expression was then observed by fluorescence microscope at 450 – 490 nm, 400X magnification and

exposure 400 ms. GFP intensity was measured semi-quantitatively using ImageJ software.

Growth Characterization of Co-transformed *Escherichia coli* BL21(DE3)

Growth characterization of Co-transformed *Escherichia coli* BL21(DE3) was determined using the growth curve with standard curve which previously made. The standard curve was made by the determination of 5 OD points: 0,1; 0,3; 5; 0,7; 0,9. For each OD value, we performed serial dilution and Total Plate Count (TPC). Standard curve was constructed by making the correlation between OD values in the y-axis with the number of cells in CFU/ml at the x-axis [10]. Constructing a growth curve, a single colony picked and grown as a liquid culture for first activation. 5 % of culture solution was inoculated into 50 ml fresh LB containing 50 µl chloramphenicol and 50 µl kanamycin. The construction of growth curve was done by measuring OD600 for 13 points with an interval of measurements every 2 hours. A total of 2 ml culture at every point put into clearsterile cuvette and the OD was measured by UV-Vis spectrophotometer at a wavelength of 600 nm. Growth curve was made by correlating log of the number of cells (Y axis) at any particular time (X axis).

AFB₁ Exposure Test in Co-transformed *Escherichia coli* BL21(DE3)

AFB₁ exposure test was conducted during the peak of exponential phase biosensors. A total of 10 ml liquid culture biosensors was exposed by AFB₁ concentrations of 0 ppb (control), 2 ppb, and 20 ppb. Observations were done at 0 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes for each concentration. Observation was including GFP expression and growth measurements at OD600. GFP expression was observed by fluorescence microscopy at 450-490 nm with the magnification of 400X. GFP intensity was measured semi-quantitatively using ImageJ software.

RESULTS AND DISCUSSION

Confirmation of pKCYP and PSOSGFP

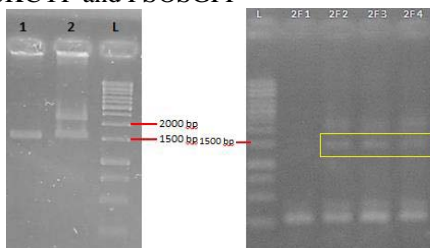


Figure 1 Confirmation of pKCYP plasmid (a) restriction pKCYP with *EcoRI* and *PstI*; (b) PCR pKCYP with VF2 and VR primers

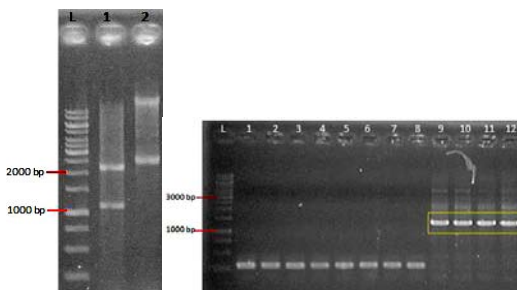


Figure 2. Confirmation of pSOSGFP plasmid (a) restriction pSOSGFP with *EcoRI* and *PstI*; (b) PCR pSOSGFP with primers VF2 and VR

The result from restriction digest plasmid pKCYP, obtained 2 band at 1500 bp and 2000 bp. Band 1500 bp was pKCYP insert composed of constitutive promoter, RBS, CYP 3A4 coding sequence, and terminator. While band 2000 bp was a plasmid backbone. For PCR pKCYP, there was a thin band at 1500 bp, show that insert pKCYP have succesfully amplified. The result from restriction digest plasmid pSOSGFP, obtained 2 band at 1100 bp and 2200 bp. Band 1100 bp was pSOSGFP insert composed of promoter SOS (*recA*) and composite GFP (RBS, GFP coding sequence, terminator). While band 2200 bp was a plasmid backbone. For PCR pSOSGFP, there was band between 1000 bp and 1500 bp, shown that insert pSOSGFP have succesfully amplified.

SOS Promoter Test

In SOS promoter test, *E. coli* BL21 (DE3) containing pSOSGFP were exposed to UVC. The purpose use of UV C with a wavelength 100-280nm was to damage the DNA of bacteria, because the absorption maximum for DNA and RNA was at 260 nm. UV radiation damages DNA bacteria by forming pyrimidine dimers, causing pyrimidine bases on the same DNA strand become covalently bonded to each other [10].

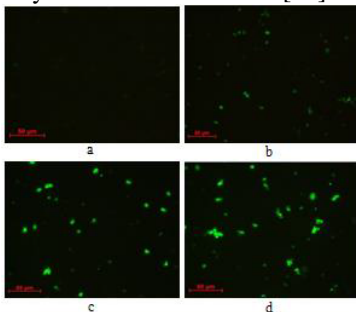


Figure 3. Expression of GFP was observed with a fluorescent microscope (excitation 450-490 nm), UV exposure for (a) 0 min (control); (b) 30 min; (c) 60 minutes; (d) 90 minutes.

After measuring qualitatively at 0 min UV exposure, it looks very faint green fluorescence. In the 30-minute exposure, it becomes more visible green fluorescence, yet the cells were exposed during 60 and 90 minutes become clearer green fluorescence than the other treatments. GFP expression was then measured semi-quantitatively using ImageJ software.

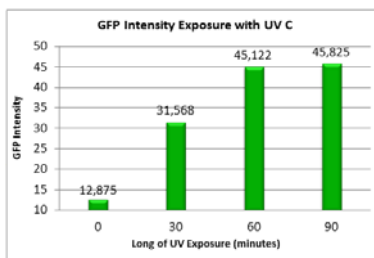


Figure 4. Semi-quantitative measurement of GFP intensity in SOS promoter test.

According to semi-quantitative measurement, control was exposed to UV showed GFP expression at 12.875. It is possible that its expression is basal expression of GFP. On exposure of 30 minutes, 60 minutes, and 90 minutes, respectively for 31,568; 45.122; 45.825. Fluorescence generated at 60 and

90 minutes exposure virtually indistinguishable. This is possible due to the UV exposure of 90 minutes, many cells have been damaged, thus reducing the overall number of cells which able to express GFP. Expression of green fluorescence of GFP showsthat SOS promoter can respond to DNA damaged.

Growth Characterization of Co-transformed *Escherichia coli* BL21(DE3)

Growth characterization was done by making the standard curve and growth curveof Co-transformed *Escherichia coli* BL21(DE3). According to standard curve, obtained equation which correlating OD value and the log cell number, namely $y = 0,2938x+8,529$ with $R^2 0,9717$.

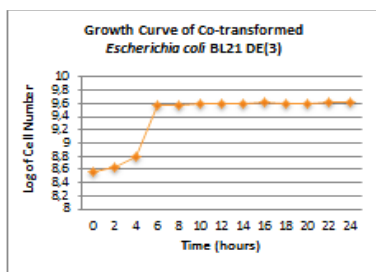


Figure 5. Growth Curveof Co-transformed *Escherichia coli* BL21 DE(3)

According to standard curve,exponential phase of co-transformed *E. coli* BL21 (DE3) was started in the first 2 hours. It can be shown from the curve that the number of cells was started to increase, from $8,610 \times 10^7$ CFU / ml to $1,135 \times 10^8$ CFU / ml. The peak of exponential phase occurs at 4 to 6 h after inoculation, during 120 minutes. The cell number in the peak of exponential phasearound $1,974 \times 10^8$ - $9,119 \times 10^8$ CFU / ml.The rate of exponential growth is influenced by environmental conditions such as temperature, composition of the culture medium, and genetic characteristics of these organisms [10]. In the biosensor, the genetic characteristics of an organism is influenced by plasmid pKCYP and pSOSGFP. This is because the plasmid is a metabolic burden to the cells. Aflatoxin-stationary phase of Whole Cell Biosensor observed 6 hours after inoculation, and lasts until the end of the observation period. Stationary phase generally occurs when the nutrients in the medium has been used, or during the metabolites products of organisms accumulate in the medium and inhibit the growth of these organisms [10].

AFB₁ Exposure Test in Co-transformed *Escherichia coli* BL21(DE3)

According to growth curve, known the peak of exponential phase occurs at 4 to 6 hours after inoculation, and occurred during the 120 minutes. AFB₁

exposure test performed during that phase, with observation time at 0 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes.

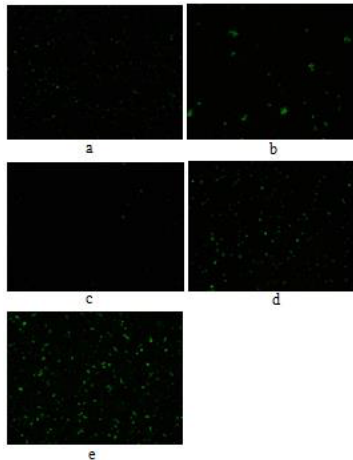


Figure 6 AFB₁ exposure test on 2 ppb, during (a) 0 min; (b) 30 min; (c) 60 minutes; (d) 90 minutes; (e) 120 min

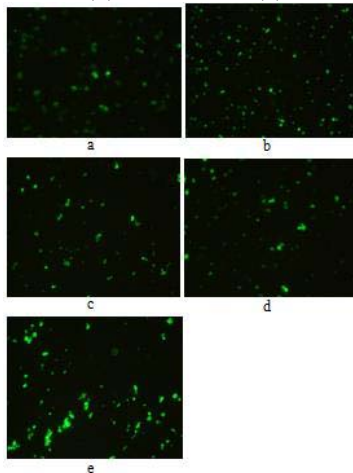


Figure 7 AFB₁ exposure test on 20 ppb, during (a) 0 min; (b) 30 min; (c) 60 minutes; (d) 90 minutes; (e) 120 min

According to AFB₁ exposure with three variations of concentrations, the brightest GFP expression results from 20 ppb exposure of AFB₁, followed by 2 ppb and 0 ppb (control). It is possible due to the exposure of 20 ppb

AFB₁. DNA damage mostly occurs, so GFP under the regulation of the SOS promoter was greatly expressed. In control, which is not exposed, there is a green fluorescence indicates that GFP is expressed. Possibility of GFP expression in control is a GFP expression in basal level.

At exposure concentrations 20 ppb, the highest GFP intensity resulting from an exposure time of 120 minutes, amount 53.406. At exposure concentrations of 2 ppb, the highest GFP intensity resulting from the exposure time for 120 minutes amount 34.9962. While at the concentration 0 ppb, the highest GFP intensity resulting from the exposure time for 90 minutes, amount 30.7659. For each concentration of AFB₁ exposure, GFP intensity patterns tend to be irregular with long exposure. Possibility is due to the result from unequal pSOSGFP segregation pSOSGFP to daughter cells during cell division. Moreover, measurement of GFP expression were performed at the peak of the exponential phase, in which the rate of cell division is at the highest level.

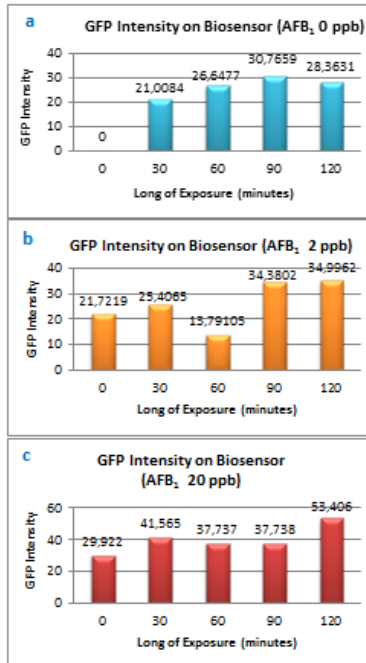


Figure 8 semi-quantitative measurement of GFP intensity of Biosensor (a) 0 ppb; (b) 2 ppb; (c) 20 ppb

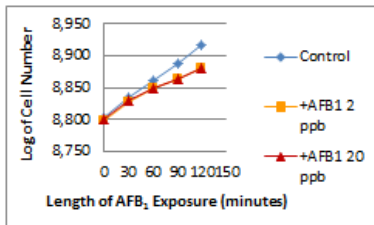


Figure 9 Relationship between logarithmic value of biosensor cell number with length of AFB₁ exposure.

The effects AFB₁ exposure on biosensor cell growth was also observed. According to the curve, the biosensor which exposed to 2 ppb and 20 ppb AFB₁ encounter a slower growth compared to controls which is not exposed to AFB₁. Allegedly biosensor cells was exposed to AFB₁ undergo DNA damaged, thereby inhibit biosensors cell replication and cell growth indirectly.

CONCLUSION

Exponential phase of Aflatoxin-Whole Cell Biosensor based recombinant *Escherichia coli* BL21 (DE3) was started in the first 2 hours, with the peak of exponential phase occurs at 4 to 6 hours after inoculation. The cell number in the peak of exponential phase around 1.974×10^8 to 9.119×10^8 CFU / ml.

AFB₁ exposure to Aflatoxin-Whole Cell Biosensor put an effect on the intensity of GFP and cell growth. In GFP intensity, increased concentrations of AFB₁ will increase the intensity of GFP. Moreover, AFB₁ exposure to Aflatoxin-Whole Cell Biosensors can inhibit cell growth.

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