Engineering of Bacterial Cell for Whole Cell Phenol Biosensor Applications
การดัดแปลงเซลล์แบคทีเรียเพื่อใช้ในการตรวจวัดสารประกอบฟีนอล

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ABSTRACT

Phenol compounds are generally known to be hazardous substances that have been defined the regulated level of its contamination in many sources. Current phenol compound detections mainly rely on chromatographic methods, that are complicated and expensive. Substitute the current methods is challenging. In this study, we have developed whole-cell biosensor based on reaction cascade to generate bioluminescent signal. The reaction cascade composed of flavin-dependent dehalogenase (HadA), flavin reductase (C_1) and firefly luciferase (Luc) enzymes. To construct whole-cell biosensor, three genes, including *hadA, c1* and *luc* were assembled using the Gibson assembly kit. The constructed plasmid was transformed and expressed in *E. coli* BL21 (DE3) with ZY auto-induction media at 25°C. The cascade enzyme expressing cell could uptake *para*-nitrophenol (4-NP) and sense 4-NP by producing a detectable bioluminescent signal in the presence of ATP, D-cysteine and $MgCl_2$. The results demonstrate that the cell harboring cascade enzyme can be used to detect the 4-NP compound.

บทคัดย่อ

สารประกอบฟีนอลเป็นที่รู้กันโดยทั่วไปว่าเป็นสารอันตรายที่ได้รับการกำหนดระดับการปนเปื้อนในแหล่งต่างๆ วิธีการตรวจหาสารประกอบฟีนอลในปัจจุบันจะใช้วิธีทางโครมาโทกราฟิค ซึ่งเป็นวิธีที่ซับซ้อนและราคาสูง ดังนั้นการพัฒนาวิธีการตรวจเพื่อทดแทนวิธีปัจจุบันจึงเป็นสิ่งที่น่าสนใจ ในการศึกษานี้เราได้พัฒนาระบบตรวจวัดแบบ whole cell โดยอาศัยหลักการของปฏิกิริยา enzyme cascade เพื่อให้ได้สัญญาณแสง bioluminescent ซึ่งปฏิกิริยาจะประกอบไปด้วยเอนไซม์ flavin-dehalogenase (HadA) flavin reductase (C1) และ luciferase จึงได้มีการสร้างระบบตรวจวัดนี้ขึ้นโดยการนำยีนของ enzyme cascade ทั้งสามตัวได้แก่ *hadA, c1* และ *luc* มาประกอบเข้าด้วยกันโดยใช้ชุดคิท Gibson assembly จากนั้นยีนของ enzyme cascade ได้ถูกนำเข้าและแสดงออกในเซลล์ *E. coli* BL21 (DE3) ในระบบอาหารเลี้ยงเซลล์แบบ ZY auto-induction ที่อุณหภูมิ 25 °C ซึ่งเซลล์ที่แสดงออก enzyme cascade นี้สามารถดูดซึม 4-NP เข้าไปภายในเซลล์และตรวจวัด 4-NP โดยการให้สัญญาณแสง bioluminescent ได้ในสภาวะที่มี ATP D-cysteine และ $MgCl_2$ จากผลการทดลองแสดงให้เห็นว่าเซลล์ที่มี enzyme cascade สามารถใช้สำหรับตรวจวัด 4-NP ได้

Keywords: Bioluminescence, Whole cell biosensor, Phenol detection
คำสำคัญ: การแปล่งแสงลูมิเนสเซนซ์ ตัวตรวจวัดแบบใช้เซลล์ การตรวจวัดสารประกอบฟีนอล

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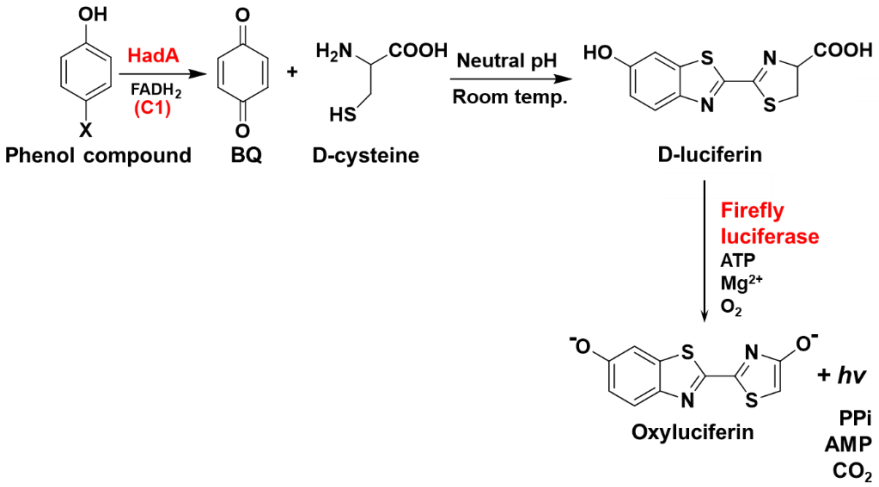
Introduction

Many phenol compounds, nitrophenols (NPs) (WHO, 2000) and halogenated phenols (HPs) (ATSDR, 1999) are classified as hazardous substances which is regulated the contaminated level in environment, water reservoir and foods by USEPA (Ghosh A et al., 2010). Phenol compounds are widely used as a precursor for synthesizing dyes, pesticides, pharmaceuticals, explosives, and solvents (Yi S et al., 2006, Kalyoncu S et al., 2016). Moreover, breakdown of pesticides, which are used to be a crop protector, also results of phenol compounds generation, i.e. 4-nitrophenol (4-NP) from parathion degradation (Wong JM et al., 1991). The over manufacturing of phenol and overuse and misuse of pesticides are major causes of their environmental contamination which can lead to adverse effects on animal and human health. The consumption of phenol compounds that are pesticide derivatives can lead to acute cholinergic crisis and diseases such as motor neuron (MND), Parkinson's and cancer diseases (Kanavouras K et al., 2011).

The residue of the phenol compound is regulated by many organizations to control the dose to be less than the level that potentially causes human health, European Unit (EU) and United States (US) (Igbinosa EO et al, 2013). This has been turned out to be a standard policy used by government officers to rule the phenol contamination level in many sources. Phenol compound detection has become an important method to controls and acts as a means of safety sources and foods guarantee. The existing accurate analytical methods of phenol compounds are HPLC and GC coupled with UV or MS detectors (Heberer T and Stan HJ., 1997, Zhang H et al., 2010, Pan Y and Zhang X., 2013). These techniques are high efficiency, sensitivity and specificity. However, they are expensive, complicated to operate and time-consuming process. Several accessible technologies that develop detection methods possessing a simple, portable process and can be applied on-site detection, have recently been established (Fukumori F and Hausinger RP, 1993). In this study, we aimed to develop phenol compound detection using light generating enzyme cascade to offer the phenol compound detection in environment, water reservoir and agriculture samples without sample pretreatment. From the chemo-enzymatic cascade reaction based on flavin-dependent dehalogenase (HadA) and firefly luciferase (Luc) to generate bioluminescent signal proposed by Watthaisong P et al., (2019) shown in Scheme 1, we have applied this cascade principle to be a whole cell biosensor. In this cascade, HadA catalyzes the degradation of phenol compound with the requirement of C1 enzyme activity to serve $FADH_2$ substrate, to yield benzoquinone. Then, D-cysteine incorporates with benzoquinone to produce D-luciferin that will be catalyzed by Luc to generate the detectable light signal. Three genes of enzyme cascade were assembled to construct and express the in a whole cell. The 4-NP could enter into the cell and the chemo-enzymatic cascade sequentially converted 4-NP to generate Luc substrate, D-luciferin, and the bioluminescence signal is a representative for phenol existing in the sample.



Scheme 1 Enzyme cascade reaction for the 4-NP detection.

Objectives of the study

To construct and develop whole-cell biosensor for phenol compound detection.

Methodology

Plasmid construction

To construct *E. coli* cell to carry genes of enzymes in the designed cascade, three genes, *hadA*, *c1* and *luc* genes were assembled in pET11a expression plasmid (Figure 1). The plasmid containing three inserted genes has size ~9,818 bp. The overlapping forward and reverse primers of all assembled genes were designed using NEBuilder Assembly Tool 2.0 (Table 1) for gene amplification. Inserted genes were amplified with denaturation temperature 98 ºC, annealing 60 ºC and extension at 72 ºC for 30 cycles.

To assemble the genes, 0.2 pmol of three inserted genes were incubated at room temperature for 15 minutes before adding of 0.07 pmol of *Nde*I excised pET11a plasmid, and the volume was adjusted to 10 µL. Then, add 10 µL of 2X Gibson assembly Master Mix reagent (New England BioLabs, Ipswich, Massachusetts, US) to 1X final concentration. The reaction mixture was then incubated at 50 ºC for 90 minutes. The assembled reaction was then transformed into *E coli* XL1blue and grown on LB agar containing 100 µg/ml ampicillin. The colonies on LB agar plate were picked into 5 ml of LB medium containing 100 µg/ml ampicillin, and cultured at 37°C for overnight. Then, the plasmids were extracted using alkaline lysis method and cut with *Kpn*I and *Nco*I restriction enzymes to check the corrected of assembled gene (Figure 2) and expected products were ~8.0 kb and ~ 1.8 kb on agarose gel.

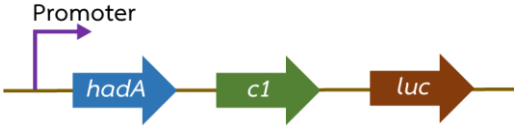


Figure 1 Gene assembly for phenol compound detection system.

Table 1 Overlapped forward and reverse primers of gene amplification.

Primer name	Primer 5' (overlap/spacer/ANNEAL) 3'
*hadA*_forward	5’ ctttaagaaggagatatacaATGATTCGTACCGGCACG 3’
*hadA*_reverse	5’ ATTGATTCATATGTATATCTCCTTTCAAATCGCCATGGCGCC 3’
*C1*_forward	5’ ggcgatttgaaaggagatatacatATGAATCAATTAAATACAGCTATTG 3’
*C1*_reverse	5’ CATCTTCCATATGTATATCTCCTTTTAAATACCAATCAGATCCTTG 3’
*luc*_forward	5’ tggtatttaaaaggagatatacatATGGAAGATGCTAAAAATATCAAG 3’
*luc*_reverse	5’ tcgggctttgttagcagccgTTACACCGCAATTTTTCC 3’

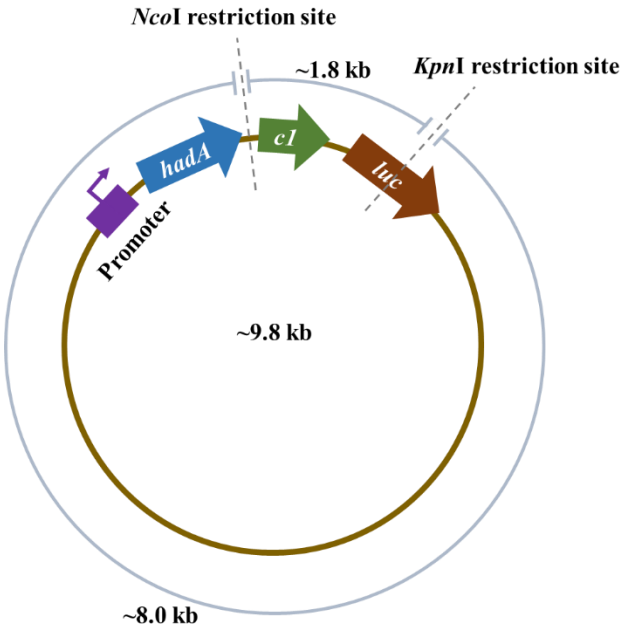


Figure 2 pET11a containing hadA, c1 and luc assembled plasmid. Correct assembly of hadA, c1, and luc genes in pET11a plasmid provided ~9.8 kb plasmid. KpnI and NcoI digestions could result ~8 and ~1.8 kb fragments.

Enzyme cascade expression in engineered *E. coli* for phenol detection.

The cascade enzyme expression was investigated in two expression media, auto-induction (ZY) and Luria-Bertani (LB)-IPTG media. The constructed plasmid was transformed into *E. coli* BL21 (DE3) and grown on LB agar containing 100 µg/ml ampicillin for starter culture. The bacterial cells in starter culture were 1.0% (v/v) inoculated in two media, ZY and LB. Then, they were grown at 37°C until the optical density at 600 nm (OD_{600}) reached around 1.0. For auto-induction media, the culture temperature was switched to 25 °C for 16 h to allow protein expression. While in Luria-Bertani (LB) broth, 1 mM of IPTG was added and protein expression proceeded for 16 h at 25 °C. Cells were harvested using centrifugation, 8,000 rpm at 4°C for 10 min. The cell paste was resuspended with 50 mM sodium phosphate buffer pH 7.0 and disrupted by sonication using Sonic Vibra_cellTM; model VCX750. Cell debris was removed by centrifugation at 15,000 rpm at 4 °C for 1 h. The supernatant was indicated as a crude extract and verified the expression efficiency by SDS-PAGE electrophoresis.

4-NP consumption by whole cell biocatalyst

To test whether the phenol compound can enter into the engineered *E. coli* cell, the 4-NP was used as a model. The experiment was performed by transforming the assembled plasmid into *E. coli* BL21(DE3) and protein expression was followed the condition from previous concluded result. The increment step is the adding of 0.2 mM 4-NP after the culturing of cell at OD_{600} reach to 1.0. Then, the expression temperature was switched to 25 °C. Cell growth and 4-NP consumption were monitored at wavelength 600 and 320 nm, respectively.

Whole cell biosensor for 4-NP detection

To investigate whether the whole cell could provide the light signal in the presence of 4-NP, cell was firstly prepared. The expression of enzymes in the cascade in whole cell was performed same as previous experiment. The cell that contained only *luc* gene was used to be a control cell to find the background of bioluminescent intensity. After harvest cell, the cell paste was washed by 100 mM HEPES pH 8.0 buffer. The starting cell OD_{600} ~10 was maintained with 1 mM ATP, 1 mM D-cysteine and 1 mM $MgCl_2$ at room temperature for 5 minutes. Then, 0.75 µM 4-NP was injected into whole cell containing cascade enzyme and only Luc enzyme. Light signal was measured by AB-2270 Octa Luminescencer. The experiment was performed in triplicate and data were presented as mean±SD.

Results

A whole cell biosensor is considered to be a cheap system due to some substrates and co-enzymes required for the detection reaction exist inside the cell. Therefore, it is not necessary to supply additional expensive reagents. We took this advantage by establishing the whole cell biosensor for pesticide detection. The concept herein of whole cell phenol detection was based on the cascade reaction of HadA chemo-enzymatic reaction and Luc. The genes of enzymes involved in HadA chemo-enzymatic cascade were assembled according to the Gibson assembly method (See Methodology). For

detection, the constructed plasmid was then transformed into *E. coli* BL21(DE3) cell to express enzymes. In the presence of phenol, expressed enzymes enabled cell to convert phenol and yield D-luciferin, a substrate of Luc that is responsible for generating the bioluminescent signal (Figure 3).

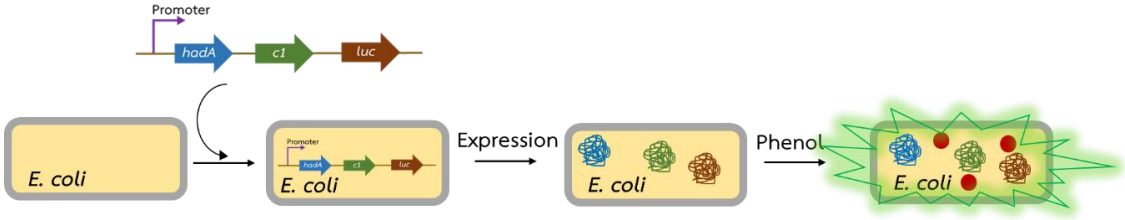


Figure 3 Engineering and expression of genes of cascade enzymes in whole-cell biosensor.

Gene construction of pesticide detection system

Three genes, *hadA*, *c1* and *luc* were assembled in pET11a plasmid (9,818 bp) using Gibson assembly kit. The constructed plasmids were transformed into *E. coli* XL1blue and six colonies were selected. Then, plasmids were extracted using the alkaline lysis method, before being cut with *Kpn*I and *Nco*I restriction enzymes and analyzed by agarose electrophoresis. The results in Figure 4 showed agarose electrophoresis analysis of uncut and cut of clone number 1, 2 and 4. For cut plasmid, only clone number 4 exhibited the expected two bands due to double digestion of *Kpn*I and *Nco*I restriction enzyme, which represented in 8.0 kb and 1.8 kb bands. This suggested that the clone number 4 contained desire assembled plasmid. This plasmid was send for DNA sequencing to confirm the correctness of assembled genes (Data not shown).

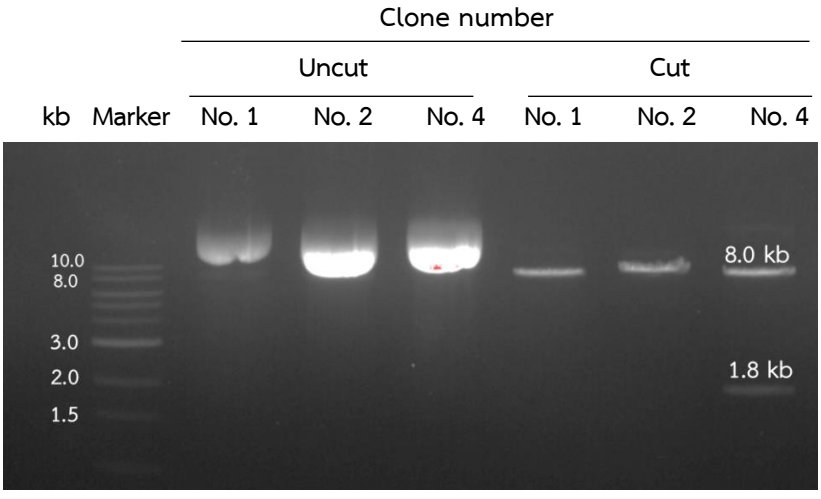


Figure 4 Agarose gel electrophoresis analysis of assembled genes by Gibson's assembly. The assembled genes were cut using *Kpn*I and *Nco*I restriction enzymes at 37 ºC for 3 h.

Enzyme cascade expression in engineered *E. coli* for phenol detection.

The expression of enzymes in assembling constructs was examined in *E. coli* BL21 (DE3) using auto–induction media and LB-IPTG systems. Expressed cells were disrupted and then crude extracts were subjected to SDS-PAGE analysis. As shown in Figure 5, LB-IPTG media was not suitable for enzyme expression due to most of expressed enzymes were insoluble form (LB pellet). In ZY auto–induction media (ZY supernatant), showed better expression of three enzymes with soluble forms, which showed the size of Luc of ~61 kDa, HadA and C_1 of 58 and 32 kDa, respectively. And the sizes were consistent to control loading of purified enzymes. Therefore, the enzyme cascade for phenol detection was successfully expressed in *E. coli* BL21(DE3) using ZY auto–induction media with 1% inoculation at 25 °C for 16 h condition.

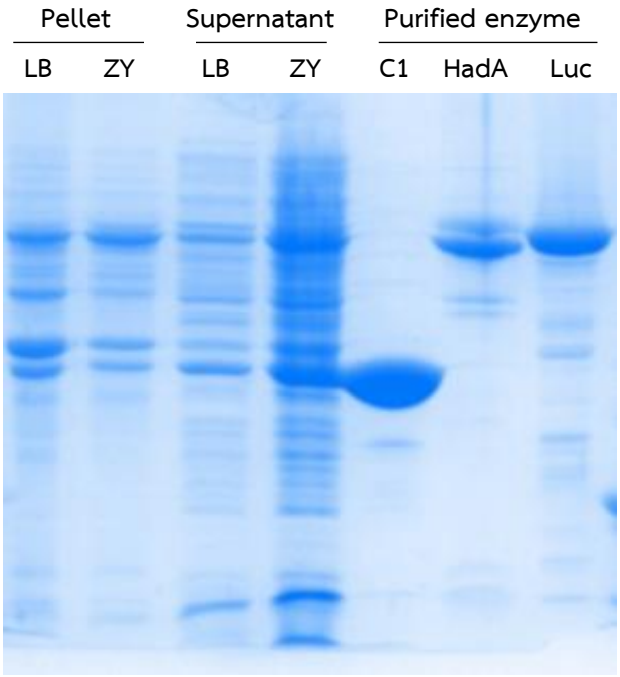


Figure 5 SDS-PAGE analysis of cascade enzyme expression using auto–induction and LB-IPTG media with 1% inoculation at 25 °C for 16 h.

4-NP consumption by whole cell biosensor

The 4-NP uptake was examined in engineered *E. coli* BL21(DE3) cell. The result in Figure 6 showed that the growth rate at 25 °C was slower than those of 37 °C (blue line), while the uptake of 4-NP at 25 °C was faster and completed within 20 h (yellow line). The pH of media was measured before and after culturing and showed the pH around 6.2-6.3. So, at 25 °C the whole-cell biosensor could completely take 4-NP into the cell.

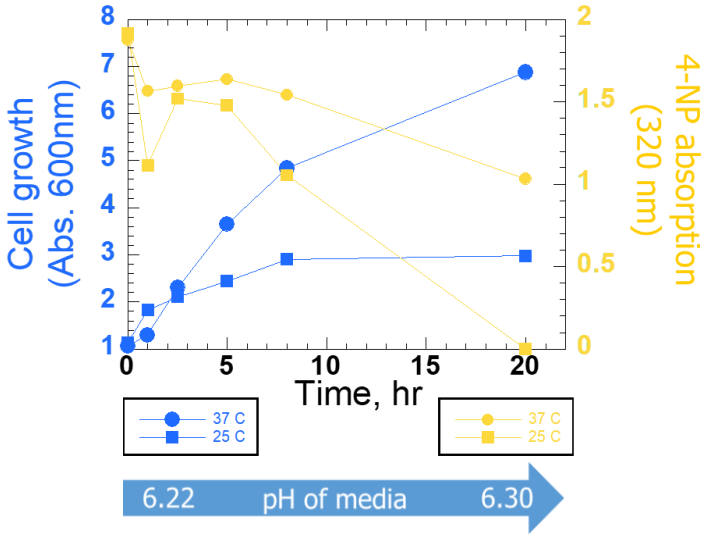


Figure 6 The growth rate at 37 °C (circle blue) and 25 °C (square blue) and 4-NP consumption at 37 °C (circle yellow) and 25 °C (square yellow) of *E. coli* biosensor cell.

Moreover, the toxicity test of 4-NP on the constructed cell in Figure 7 shows the effect of 4-NP concentration on cell growth as monitored at OD_{600}. The results showed that the concentration of 4-NP higher than 0.16 mM affected on cell growth. This suggests the suitable working concentration of 4-NP should be lower than 0.16 mM.

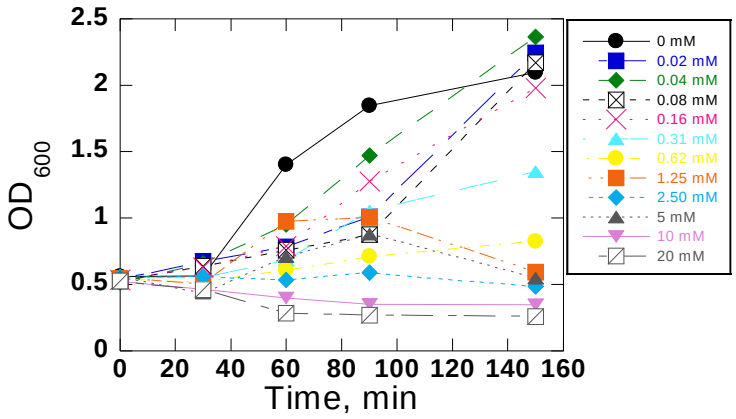
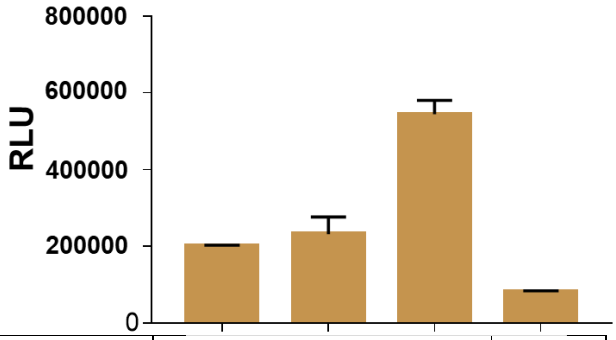


Figure 7 The growth rate of cascade enzyme expressing cell in the presence of various concentrations of 4-NP.

Whole-cell biosensor for 4-NP detection

The ability of constructed cell to sense the 4-NP compound was investigated. The enzyme cascade expressing cell was prepared and incubated with 4-NP compound. The result in Figure 8 demonstrates that *E. coil* cell expressing the cascade enzymes could generate light signal in the presence of 4-NP and require the exogenous addition of ATP, D-cysteine and $MgCl_2$. Without adding of ATP, the light signal was not significant difference to the negative control, in the absence of 4-NP. While the cell that contained only Luc showed light signal as a background control. This suggests that the constructed biosensor cell could take 4-NP and 4-NP could be converted by enzymes in the designed cascade to form the D-luciferin and provide light signal. The exogenous adding of ATP, D-cysteine and $MgCl_2$ were crucial for this whole-cell biodetection.



Components	HadA-C1-Luc			Luc
4-NP (μM)	-	0.75	0.75	0.75
ATP (mM)	1	-	1	1
D-cysteine (mM)	2	2	2	2
$MgCl_2$ (mM)	1	1	1	1

Figure 8 The bioluminescent signal in the presence and absence of the target compound, 4-NP emitted from whole-cell biosensor. The cell expressing only Luc enzyme was used to be background control.

Discussion and Conclusion

The *E. coli* cell was engineered to carrying genes of cascade enzymes for phenol compound detection. Three genes assembly was constructed using Gibson assembly strategy (Gibson DG, 2011). This assembly method can be used to construct synthetic genes, genetic pathways, and entire genomes. The ZY auto–induction media at 25 °C was suitable for expressing of cascade enzymes. Even though the less expression of each enzyme was shown in SDS-PAGE analysis, but the activity of the expressed enzyme was adequate for converting the 4-NP to quinone and ultimately produced D-luciferin (Figure 8). The result in Figure

6 indicated that whole-cell biosensor could take high level of 4-NP (0.2 mM). This is due to a small size and highly soluble in water of 4-NP, leading to a simple diffusion by accumulating in the hydrophobic regions of lipid bilayer through hydrophobic interaction to penetrate into cells (Pandey G et al., 2003, Zhang R et al., 2019). The results in Figure 8 suggested that after taking 4-NP into the cells, the 4-NP was subsequently converted to produce D-luciferin that served as a substrate for Luc to generate light signal. In addition, this system required exogenous compounds, including ATP, D-cysteine and $MgCl_2$ for the detection. ATP is an organic compound that provides energy to maintain biological activities of living cells. Moreover, in this enzyme cascade, it serves as a Luc substrate for the formation of luciferyl adenylate which is an intermediate in the bioluminescent reaction (Ford SR et al., 1996). While the extracellular D-cysteine was also required for this whole- cell biosensor. This is because D-isomer of cysteine amino acid does not mainly exist in cell in general (Niwa K et al., 2006). The D-cysteine is indeed needed for unanalyzed reacting with quinone, the resultant product from HadA reaction to form D-luciferin. Although, Mg^{2+} ion is the trace element that should exist in bacterial cells. In this biosensor case, the Mg^{2+} ion concentration may not be enough to be a cofactor to support Luc activity (DeLuca M and McElroy WD, 1974).

In summary, the engineering of *E. coli* BL21 (DE3) to carrying cascade enzymes for phenol compound detection was successfully constructed. This whole-cell biosensor was able to sense the 4-NP as it could provide the detectable bioluminescent signal in the presence of 4-NP. Further studies are required to observe the quantitative detection and determine the detection limit of this whole-cell biosensor. This result will pave the way to develop a powerful biosensor that is easy to operate, portable and inexpensive for primary screening of contaminated phenol compounds in several sources.

Acknowledgement

The authors thank Mahidol University (to PK and RT), Vidyasirimedhi Institute of Science and Technology (to PW and PC), the Central Instrument Facility (CIF), Faculty of Science, Mahidol University (to RT), Enzmart Biotech co., ltd. for the technical support.

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