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Homogentisic acid-based whole-cell biosensor for detection of Alkaptonuria disease

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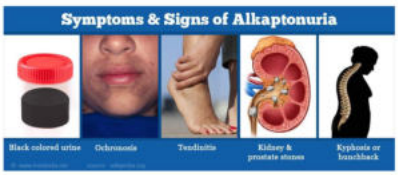
Introduction:

- **Alkaptonuria** is an autosomal disease caused by a deficiency of the enzyme homogentisate 1,2 –dioxygenase.
- This enzyme deficiency results in an increased level of **homogentisic acid**
- This disease is usually not diagnosed until the patient presents with progressive premature **osteoarthritis** as a young adult, currently **no cure** for alkaptonuria.
- Phe and Tyr restricted diets and ascorbic acid supplements are given to patients of alkaptonuria. Thus, continuous monitoring of homogentisic acid can help maintain stringent diet.
- Analytical techniques like enzymatic spectrophotometry, gas chromatography, and ultra-high-performance liquid chromatography are the most commonly used methods for rapid and sensitive determination of homogentisic acid concentration. However, these techniques require **toxic chemicals**, expensive, highly specialized instruments with need for pre-column derivatizations which makes them **laborious**, **time-consuming**, and **cost-intensive** methods.
- In this study, we report use of a novel regulatory element isolated from *P. aeruginosa* (PAO1) genome for direct determination of homogentisic acid concentration spiked in the **urine samples** using **Whole Cell Biosensors.**
- The use of whole cells as biosensors for *in-vitro* diagnostics offers several favorable characteristics such as low cost of production, **sustainable product**, the ability for point of care device development, self-replicating ability.
- We utilized signaling pathway to construct a biosensor for the detection of alkaptonuria by transcriptionally fusing the upstream genetic regulatory promoter region of ***hmgA*** to the green fluorescence protein (GFP) gene in *P. aeruginosa* chassis.



Literature:

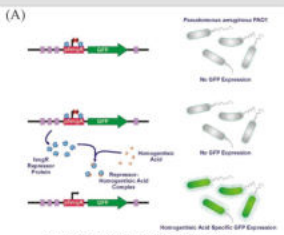


Figure (A). Schematic illustration of sensing module

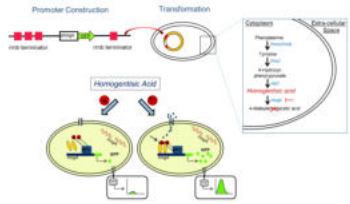


Figure (B). Schematic illustration representing whole-cell biosensor for the detection of homogentisic acid in alkaptonuria patients.

- *P. aeruginosa* metabolizes Phe and Tyr through a peripheral pathway involving homogentisic acid as a central intermediate.
- We utilized this signaling pathway to construct our biosensor by transcriptionally fusing the promoter region of hmgA to green fluorescence protein (GFP) in wild type strains and various transposon mutant.
- The sensor construct was further checked for analytical features such as sensitivity, linearity, selectivity, and precision for quantitative detection of homogentisic acid and to check the clinical utility of the purposed sensor construct for detection of alkaptonuria patients.

Methods:

Bacterial strains and plasmid

- *P. aeruginosa* PAO1, MPAO1, and various MPAO1 transposon mutants were used as a host for the plasmid-borne transcriptional fusion of *hmgA* promoter sequence with green fluorescence protein (GFP).
- Plasmid **pPROBE-NT** was used as a base vector.
- Overnight cells were inoculated in 3 mL of Luria broth (LB) with 50µg/ml of neomycin under shaking conditions at 180 rpm at 37°C.

Construction of the biosensing module

- The upstream region of genetic locus coding for homogentisate dioxygenase gene *hmgA* was introduced into the forward primer that together with reverse primer was used to amplify the promoter sequence of *hmgA* gene from the genomic DNA of *P. aeruginosa* (PAO1).
- Unique restriction sites (*Eco*R1 and *Hind*III) were introduced in the primers for upstream and downstream regions of the amplified fragment.

Measurement of fluorescence intensity

- 100 µl of the diluted cell culture was then added to the clear bottom 96-well microtiter plate. The plate was incubated at 37°C for 12 h during which fluorescence (485nm excitation/535nm emission) and absorbance (600nm) were measured at 30 min intervals using a microplate reader.

Figure (D). Construction of phmgA plasmid using restriction enzyme *Eco*RI and *Hind*III

Evaluation of Cross-reactivity and linearity

- To verify the specificity of the *phmgA::GFP* based reporter system for homogentisic acid, we examined the fluorescence response of the proposed biosensor with twenty amino acids at a concentration of 160µM. **No Cross-reactivity was reported.**

Analysis:

- All data were plotted, and statistical analysis was conducted with GraphPad Prism software. The lower limit of detection was calculated using the formula from the limit of background (LOB) value ($Mean_{(blank)}$ +1.645 X $SD_{(Blank)}$) using the equation LLOD = LOB + 1.645 X $SD_{(lower\ concentration\ sample)}$.
  - **$fold_{change}$ (inducer) = $GFP/OD_{(inducer)}$ /$GFP/OD_{(inducer\ =\ 0)}$**

Results:

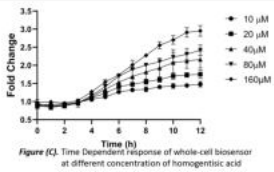


Figure (C). Time Dependent response of whole-cell biosensor at different concentration of homogentisic acid

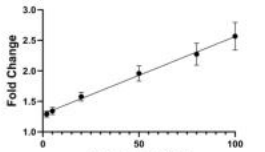


Figure (D). Linear response of homogentisic acid was determined using *phmgA::GFP* based whole-cell biosensor.

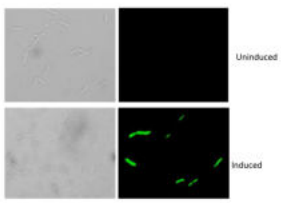


Figure (E). Flouresence imaging

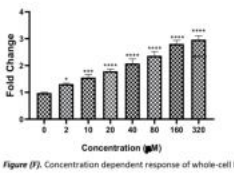


Figure (F). Concentration dependent response of whole-cell biosensor

Conclusions:

- The proposed biosensor displays excellent reproducibility, selectivity, and dynamic range (10-320µM) for homogentisic acid quantification.
- The biosensor demonstrates an improved limit of detection (3.9µM).
- This biosensor may be useful to detect other pathological conditions such as colorectal cancer, ulcerative colitis, and Crohn's disease; where the homogentisic acid levels have been associated with the disease outcome

Detection method	Limit of detection
HPLC	5µM
Color based by addition of NAOH	1mM
LC-MS/MS	62.5µM
Capillary electrophoresis method	3.3µM
LC-MS/MS	30µM
FT-IR	2mM
Cell based method (This study)	**3.9µM**

Important References:

[1] G. Jacomelli, V. Micheli, G. Bernardini, L. Millucci, A. Santucci, Quick diagnosis of alkaptonuria by homogentisic acid determination in urine paper spots, JIMD Reports, Volume 31, Springer2016, pp. 51-6.
[2] W.Y. Hsu, C.M. Chen, F.J. Tsai, C.C. Lai, Simultaneous detection of diagnostic biomarkers of alkaptonuria, ornithine carbamoyltransferase deficiency, and neuroblastoma disease by high-performance liquid chromatography/tandem mass spectrometry, Clin Chim Acta, 420(2013) 140-5.
[3] N. Oztekin, G.S. Balta, M.S. Cansever, Determination of homogentisic acid in urine for diagnosis of alcaptonuria: Capillary electrophoretic method optimization using experimental design, Biomedical Chromatography, 32(2018) e4216.
[4] A.T. Hughes, A.M. Milan, P. Christensen, G. Ross, A.S. Davison, J.A. Gallagher, et al., Urine homogentisic acid and tyrosine: simultaneous analysis by liquid chromatography tandem mass spectrometry, J Chromatogr B Analyt Technol Biomed Life Sci, 963(2014) 106-12.
[5] A.P.J.A. Markus, D.W. Swinkels, B.S. Jakobs, R.A. Wevers, J.M.F. Trijbels, H.L. Willems, New technique for diagnosis and monitoring of alcaptonuria: quantification of homogentisic acid in urine with mid-infrared spectrometry, Analytica Chimica Acta, 429(2001) 287-92.
[6] C.K. Stover, X.Q. Pham, A.L. Erwin, S.D. Mizoguchi, P. Warrener, M.J. Hickey, et al., Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen, Nature, 406(2000) 959-64.
[7] M.A. Jacobs, A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, et al., Comprehensive transposon mutant library of Pseudomonas aeruginosa, Proc Natl Acad Sci U S A, 100(2003) 14339-44.

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