Design Article

Escherichia coli Breathalyzer: Detecting Ethanol Using Transformed Bacteria

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Reviewed on 29 April 2017; Accepted on 19 June 2017; Published on 10 November 2017

Indirectly detecting blood alcohol levels both rapidly and noninvasively is key to preventing drunk driving. We propose an *Escherichia coli* based system that qualitatively reports breath alcohol concentration using green fluorescent proteins. The system would use the alcA ethanol promoter and alcR transcription factor from *Aspergillus nidulans* to trigger green fluorescent protein production in the presence of vaporized ethanol. The system would have a quantitative lower bound, over which it would report detected alcohol qualitatively. Fluorescence would be measured using either a charge-coupled device or human observation of the live cells in media.

Keywords: *Escherichia coli*, ethanol, carbon specific promoter, Green Fluorescent Protein (GFP), *Aspergillus nidulans*, biosensor

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In the United States, drunk driving accounts for almost a third of all traffic-related deaths, with more than half of all children under 14 killed in car crashes involved in some type of drunk driving (Centers for Disease Control, 2017). Although law enforcement uses breathalyzers in order to test individuals involved in drunk driving-related accidents, personal breathalyzers are not only expensive but highly inaccurate (Sy, 2009). Online, the more accurate breathalyzers can cost up to hundreds or thousands of dollars. Even so, many fail to differentiate commercial ethanol from alcohol produced from ketosis within the body, which even further skews results (Jones and Rossner, 2007). With the discrepancy between cost and effectiveness in common personal breathalyzers, we propose a system that would be not only more effective in detecting commercial ethanol, but would also be far easier to produce.

Green Fluorescent Protein (GFP) consists of 238 amino acids that are encoded by 714 nucleotides (UniProtKB - P42212 (GFP_AEQVI)). Transcription occurs at a maximum rate of 40-80 nucleotides per second at 37°C. Translation occurs at a maximum speed of 20 amino acids, or 60 nucleotides per second at 37°C. Thus, transcription of GFP would optimally take roughly 10.4 seconds to 20.8 seconds and translation would take roughly 11.9 seconds at 37°C.. The final *Escherichia coli* breathalyzer will include a heating device to maintain a temperature of 37°C to maximize transcription and translation, making GFP expression present at a minimum of 22 to 32 seconds. Therefore, although we took 24 hour data at 21.5 degrees Celsius, our device should display results in a timely manner when heated.

System Level

We propose implementing a consumer and forensic breathalyzer that utilizes the technology described below in a disposable format. The consumer device would likely contain a disposable plastic pack containing a biofilm that supports the alcA-GFP E. coli. To measure the luminance of the GFP reporter, we propose using a charge coupled device (CCD) enclosed in the device. The CCD would allow the measurement of ethanol levels, relative to luminance, without human interaction, making the device potentially easier to use. Rines, et al. (2012) describes the optical assembly necessary to perform live cell imaging measuring GFP expression levels. The device would contain a breath tube to receive the breath sample that would connect the sample to the biofilm via a one-way valve to prevent contamination. The system, after analyzing the luminance of the biofilm, would report ethanol concentration results via a screen on the device or a smartphone app.The system would consist, in total, of two parts: first, the reusable optical assembly and biofilm/sample

receptacle and second, disposable biofilms that contain transformed *E. coli* to perform the ethanol detection.

Device Level

We propose an *E. coli* K-12 system that can detect human blood ethanol levels via the alcA and alcR promoter system coupled with a green fluorescent protein (GFP) reporter downstream. The genes for alcA and GFP would be assembled onto a single ampicillin resistance carrying plasmid using BioBrick assembly, and would transform a standard inducible *E. coli* K-12 chassis using chemical poration with CaCl2. The alcR gene would be placed downstream of a constitutive promoter in the same *E. coli* containing the alcA-GFP system. This system's final, working representation as seen in the transformed *E. coli* is shown in Figure 1. This system would facilitate ethanol specific promotion of the GFP reporter.

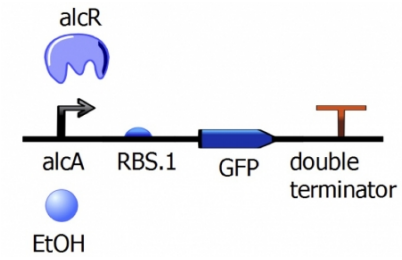

Figure 1: Graphical representation of our proposed system.

Parts Level

AlcR: This is a protein needed for the correct activity of AlcA. The gene for the expression of AlcR will be placed under continuous expression elsewhere on the plasmid.

AlcA: Alcohol dehydrogenase is the enzyme that catalyzes ethanol. AlcA is the promoter for the production of alcohol dehydrogenase. Thus, the alcA promoter is highly sensitive to differences in ethanol concentration. Transcription for alcohol dehydrogenase synthesis is strongly induced by the positive transcription regulator alcR. Primary research of alcA was conducted in *Aspergillus nidulans*. Since the alcA promoter is sensitive to nuances in ethanol concentration, alcA was selected as the promoter for the alcohol detection system. In theory, the presence of higher ethanol concentration should induce higher transcription of Green Fluorescent Protein (GFP), making *E. coli* more fluorescent; lower ethanol concentration should conversely induce lower transcription of GFP and cause less fluorescence in *E. coli*.

Ribosome Binding Site (RBS): Since we only want one RBS that is very reliable with fairly high activity, we chose iGEM part BBa_B0034, a high-efficiency RBS that is commonly used.

Green Fluorescent Protein is a protein produced by the jellyfish *Aequorea victoria*. The jellyfish has 6000-7000 photogenic cells, which emit light by a process of bioluminescence. GFP has 238 amino acids and is highly stable in neutral buffers (pH 5.5 -12) at 65°C. The protein exhibits a highly fluorescent green color and fluoresces maximally under a 400 nm light with an emission peak at 509 nm. The covalently attached chromophore is formed post-translationally within the protein when cyclisation and oxidation of residues 65-67, Ser-Tyr-Gly. In our system, transcription of GFP will begin with the presence of ethanol. Subsequently, GFP production will create fluorescence, indicating the presence of vaporized ethanol.

Double Terminator: Like the RBS, we only need a reliable terminator. Therefore, we chose IGEM part BBa_B0014, a reliable, high efficiency terminator.

Materials and Methods

Serial Dilution Experiment

We first made 100 mL LB broth from dehydrated powder. We then labelled five 10 mL Falcon tubes with dilution amounts: 95% ethanol, 9.5% ethanol, 0.95% ethanol, 0.095% ethanol, 0% ethanol. This was repeated to make blanks for spectrophotometry. We then added 10 mL 95% ethanol to both tubes labeled 95% ethanol. We also added 9 mL LB broth to all the other tubes. In the dilution step, we took 1 mL ethanol from the 95% ethanol tube and added it to the 9.5% tube. We capped and shook the recipient tube. This was repeated five times down the line - transferring from 9.5% to 0.95% and from 0.95% to 0.095%. We then made the same dilution series for the blank tube set. We finally removed 1 mL broth from both 0.095% tubes to make every sample 9 mL. We then inoculated each experimental tube with K-12 E. coli using sterile loops. We collected results after 24 hours by performing spectrophotometric analysis (with the spectrophotometer set to 600 nm). The Falcon tubes were stored in a 21.5°C environment for the entirety of the experiment.

Linear Dilution Experiment

Based on the negligible amount of *E. coli* in ethanol concentrations of 9.5%-95% from our baseline experiment, we decided to follow up the baseline experiment with a focus on the range of ethanol concentrations between 0%-9%. Although ingested alcohol concentration may be of far higher, the ethanol present in the breath after ingesting alcohol is a minute fraction of the original ingested concentrations (Jones and Andersson, 1996).

We made two sets of approximately 10.0 mL of 0%, 0.5%, 1%, 3%, 5%, 7%, and 9% ethanol and LB solutions in Falcon tubes, similar to the serial dilution. We prepared the ethanol solutions by adding the corresponding amount of ethanol (with precision of 1 µL for the 0.5% solution and 5 µL for the others) and then filling up each tube to 10.0 mL. We then capped and inverted the tubes to mix their contents. Next, we inoculated each tube of the experimental set with *E. coli* K-12 using sterile inoculating loops. Lastly, we collected 24 hour data in a maintained 21.5°C environment using the same testing procedure as the serial dilution experiment (with the spectrophotometer also set to 600 nm).

Laboratory Safety

When the ethanol solution was made for the first time, the only clean techniques that were employed were the use of sterile pipette tips, sterile Falcon tubes and clean LB broth. Little changed in the clean procedure that we used when we collected 24 hour data. We did not wash our hands, wear gloves, nor wipe down surfaces with 95% ethanol.

Results and Discussion

Absorbance is proportional to the number of bacteria in a culture (Reynolds, 2011) with a constant of proportionality that depends on the bacterial species. For *E. coli*, this constant is 8 x 10^8 cells/mL (Agilent). Therefore, cells/mL = 8 x 10^8 * Absorbance. In our first experiment, we attempted to determine the amount of ethanol in which *E. coli* can survive in ethanol to an order of magnitude (Figure 2). In our second experiment, we tried to pin down where cell survival decreased between 0.95% and 9.5% ethanol solutions.

Our data from the linear ethanol dilution experiment generally followed the expected trend of greatest bacterial growth

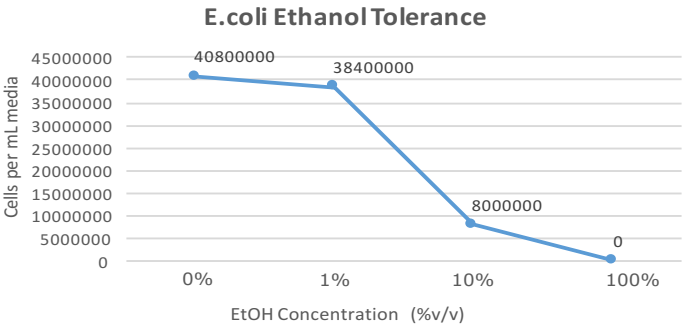

Figure 2: Linear dilution of ethanol into untransformed E. coli K-12 cultures (0% EtOH - 95% EtOH)

occurring in the 0% ethanol solution and the least bacterial growth occurring in the 7% ethanol solution (Figure 3). However, the data point for the 0.5% ethanol solution did not follow this trend. If following the trend, the 0.5% ethanol solution absorbance value was expected to be between 0.051 (1% ethanol) and 0.061 (0% ethanol). However, the actual absorbance value was 0.04, even lower than the value for 3% ethanol (0.042). In

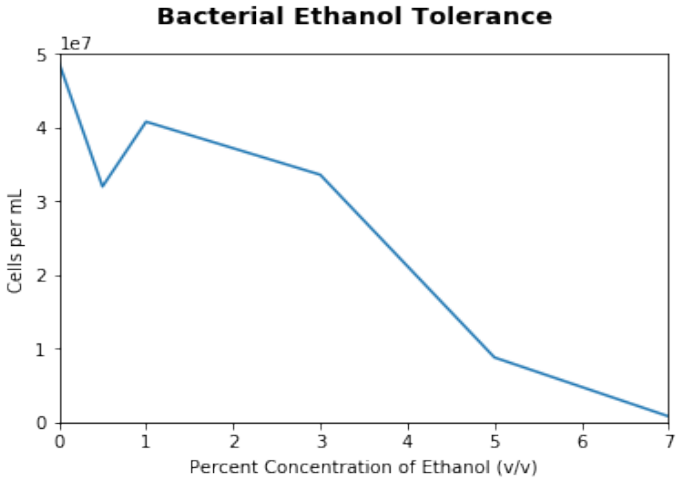

Figure 3: Stress analysis of untransformed E. coli in smaller range of ethanol concentrations (0% EtOH - 7% EtOH)

addition, the large difference from 0.011 (5% ethanol solution) to 0.042 (3% ethanol) seems to indicate that *E. coli* cannot substantially tolerate ethanol beginning around a concentration somewhere between 3% and 5%. Lastly, we are fairly certain that contamination occurred in the 0.5% ethanol blank as that particular blank was much more opaque that the 0% ethanol blank, which should be very close in opacity as the addition of ethanol does not decrease transparency since ethanol is clear. Since poor sterile technique was used while handling each ethanol solution, further contamination in other samples was also likely. Thus, further testing with better sterile technique is needed to consolidate a trend and draw a more confident conclusion.

We plan to perform an additional ethanol dilution lab with transformed *E. coli* to ascertain how well transformed *E. coli* accept their plasmid under stressful conditions. This lab will guide our final system design because it will give us a range of viability in terms of percent ethanol that is acceptable to bacterial survival.

Acknowledgements

We would like to thank the Bio Builder Foundation for their continued support, Aaron Dy, our mentor, for his help consolidating our proposed project, and Lab Central for hosting the BioBuilder final assembly presentations.

References

E. coli Cell Culture Concentration from OD600 Calculator [Internet]. Agilent Technologies, Genomics; [cited 2017 May 27]. Available from: http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp

Impaired Driving: Get the Facts [Internet]. Centers for Disease Control; 2017 Jan 26 [cited 2017 May 27]. Available from: https://www.cdc.gov/motorvehic....

Jones AW, Rossner S. False-positive breath-alcohol test after a ketogenic diet. International J Obseity. 2007 Aug 8;2007(31):559-61.

Jones AW, Andersson L. Variability of the blood/breath alcohol ratio in drinking drivers. J Forensic Sci. 1996 Nov;41(6):916-21.

Reynolds J. Lab Manual: Counting Bacteria [Internet]. Dallas (TX): Dallas County Community College District; 2011 Jan 23 [last updated 2011 Jan 23; cited 2017 May 27]. Available from: http://delrio.dcccd.edu/jreynolds/microbiology/2421/lab_manual/numbers.pdf.

Rines DR, Thomann D, Dorn JF, Goodwin P, and Sorger PK. Live Cell Imaging of Yeast. Cold Spring Harb Protoc. 2011 Sep 1;2011(9).

Redei GP. Encyclopedic Dictionary of Genetics, Genomics and Proteomics. 3rd rev. ed. n.p.: Springer Science & Business Media; 2008. 2201 p.

Sy S. More Than a Game: Testing Your Blood Alcohol [Internet]. ABC News; 2009 Dec 27 [cited 2017 May 20].

What is Faster, Transcription or Translation? [Internet]. Cell Biology by The Numbers; [cited 2017 May 20]. Available from: http://book.bionumbers.org/wha...

UniProtKB - P42212 (GFP_AEQVI) [Internet]. UniProt; 1995 Nov 1 [last updated 2017 May 10; cited 2017 May 20]. Available from: http://www.uniprot.org/uniprot...