# Project Report


## *Don't let waste get wasted!*

# Table of Contents


1. Abstract 3
2. Introduction 3
    a. Fur transcription factor and the Fur box 4
    b. Ferric binding protein A 4
    c. Production of bioethanol 5
    d. Aggregation by biofilms 6
    e. GenBrick Assembly 7
    f. Modelling 7
3. Results 9
    a. Characterization of Bacillus subtilis 168 as a chassis 9
    b. Fur Box Assembly and Fur transcription factor assay 9
    c. Functional analysis of FbpA 10
    d. pET fusion and bioethanol 11
    e. Human practices 12
4. Conclusions 13
5. Future Work 13
6. Reference List 15
7. Appendix 1 – List of Parts Submitted 17
8. Appendix 2 – Genabler Assembly Protocol 18


Please note you can find much more information online http://2013.igem.org/Team:Edinburgh

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*The Edinburgh iGEM 2013 team consists of 9 student members (6 biologists, an engineer, an informatician and an artist), 6 instructors and 6 advisors. We would like to thank our supervisors, who had immense impact on all aspects of our project and the advisors who had to withstand our questions in the lab. Working together was a fabulous experience, which we all greatly enjoyed!*


From left to right: Jan, Gavin, Aleksandra, Kyle, Hugo, Dainius, Christiana, Harry, Weike.

# 1. Abstract

The Edinburgh iGEM 2013 team, WastED, is focusing on remediation and valorization of industrial waste streams, with a particular focus on Scottish leather and whisky industry waste waters, which contain toxic heavy metal ions as well as fermentable organic components. Using Bacillus subtilis as chassis, we are engineering organisms to capture ions using chelators and metal binding proteins, and to ferment organic components to produce biofuels. We are also testing a new assembly procedure, GenBrick, based on the Genabler assembly system. GenBrick allows assembly of multiple RFC10-compatible BioBricks in a single reaction, and is also well suited to the preparation of fusion proteins and addition of terminal tags. Enzyme fusions may enhance metabolic pathways through substrate channeling. We are testing the effect of protein fusions on fermentation efficiency for biofuel production. In addition, we are examining the implications of possible Scottish independence, following the 2014 referendum, for Synthetic Biology in Scotland.

# 2. Introduction

Our aim was to create a self-contained system for bioremediation and valorization of toxic waste waters produced by the key Scottish industries. We have identified two major components of the industrial waste streams, which are believed to have the most detrimental effects on the environment: heavy metals and fermentable organic compounds.

Heavy metals can be dangerous to both health and the environment and, unlike other pollutants, they do not decay. They can lay dormant and have the potential for bioaccumulation and biomagnification. This leads to heavier exposure for some organisms, such as coastal fish and seabirds, than is present in the environment alone. Fermentable organic waste on the other hand is deleterious in a less direct way. When released to the water bodies, it can lead to occurrence of harmful algal blooms, which are of increasing concern in Scotland and worldwide through their negative effects on the biodiversity, human health and economy.

We have decided to use Bacillus subtilis as our chassis and the first step in our project was to characterize it by looking at its responses to varying ethanol and heavy metal concentrations. We then went on to create a sensory system for metal detection, followed by metal binding. We have decided to convert the fermentable organic compounds into bioethanol, which can have many potential application. As co-localization of enzymes has been previously shown to speed up metabolic pathways (see Team Slovenia 2010), we wanted to exploit this principle to increase ethanol production in bacteria by generating pET fusion protein(Whitaker et al., 2012,Conrado et al., 2012). To achieve this we have employed and tested a new assembly method, called GenBrick. Finally, we wanted to see how our manipulations might affect cell metabolism, by combining a modular model of the whole cell, to which different pathways can be slotted in.

## a. Fur transcription factor and the Fur box

In *Bacillus subtilis*, iron recognition is achieved via the Ferric Uptake Regulator (Fur). Fur normally forms a dimer and interacts with a specific operator named the fur box to repress genes further down-stream. At high intra-cellular iron concentrations, Fur binds to iron to form a holo-repressor and then binds to the fur box and represses gene transcription. Genes controlled by the fur box usually code for iron binding proteins and gate mechanisms within the cell (about 40 genes are controlled by the fur box). In the absence of significant iron levels, the apo-repressor Fur does not bind to the fur box and the relevant genes can be coded for.

There are two iron binding site per Fur, so 4 overall sites overall in the dimer. (Pohl et al., 2003) The upper binding site (S2) regularly has a zinc ion for structural integrity. The lower binding site (S1) typically has a zinc ion, which is replaced by iron when the intracellular iron concentration rises.

The fur box pattern that is conserved in all different fur boxes is the palindromic sequence: TGATAAT-N-ATTATCA (N being any base). Most fur boxes are 19 to 21 base pairs and always contain this 7-1-7 palyndromic sequence. Different theories exist as to how the Fur dimer binds to the fur box. The classical model states that a Fur dimer binds to one fur box and represses the downstream gene. Another states that the hexameric GATAAT pattern is recognized by three different fur dimers (Fuangthong and Helmann, 2003). The most recent one posits that the minimal binding site for the Fur dimer is TGATAAT-N-ATTATCA and that most fur boxes contain two repeats of this pattern, such as TGATAATGATAATCATTATCA (Figure 1).


**Figure 1.** The most recent study (Baichoo and Helmann, 2002) suggest most fur boxes have two overlapping palindromic sequence and two fur dimers bind.

We decided to assess the minimal binding site for the fur box to work (TGATAAT-N-ATTATCA) in a synthetic construct and assay the effect of iron on a fluorescent protein located downstream.

## b. Ferric binding protein A

We propose to make a system in which a metal binding protein is produced in response to the presence of that metal in the growth medium. As a proof of concept, we chose to work with iron. We planned to use the FUR box and protein to negatively regulate (in response to iron) an inverter gene, which would then positively regulate expression of an iron binding protein. Thus the more iron that is present, the more iron binding protein is produced.

We chose as our iron binding protein Ferric binding protein A (FbpA), from *Neisseria gonorrhoeae*. It is a periplasmic protein and a member of the transferrin superfamily, which binds clusters of Fe3+ (Ferreiros *et al.* 1999; Alexeev *et al.* 2003).

## c. Production of bioethanol

Microbial production of ethanol is of great importance due to its possible application as a biofuel. Increasing ethanol yields in bacteria is potentially beneficial as those are able to utilise a wider variety of renewable, biomass-derived carbon sources compared to standard ethanol producer: *Sacharomyces cerevisiae*. Our goal was to increase ethanol yields using a fusion of *Zymomonas mobilis* pyruvate decarboxylase (Pdc) and alcohol dehydrogenase B (AdhB). Reactions catalysed by those enzymes (Figure 2) enable conversion of pyruvate to ethanol.

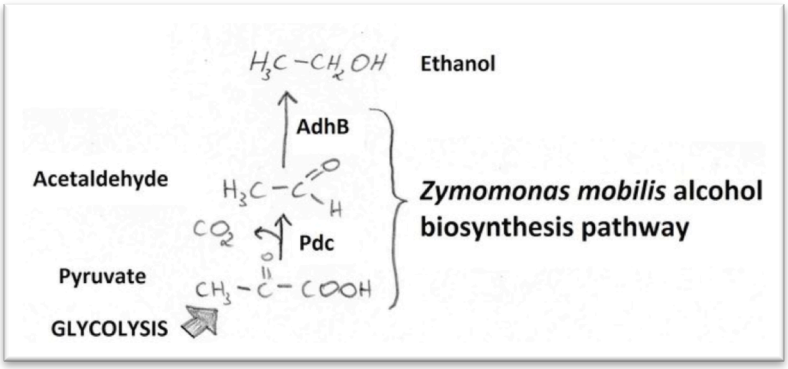


**Figure 2.** Ethanol is generated from pyruvate (fed into the pathway from glycolysis) via an Acetaldehyde intermediate.

We based the work on the hypotheses that flow of substrate from one enzyme to another should be facilitated in the fusion protein, and that due to this less of the toxic aldehyde intermediate should be released into the cell. Other fusion proteins were already presented to increase amounts of final product produced (Wang et al., 2011) what further encouraged us to undertake this work. We have decided that we will perform the fusion of C terminus of Pdc to N terminus of AdhB.

We have decided that the most accessible of the waste molecules present in the effluents from the industry would be lactic acid. This molecule is produced as a by- product of anaerobic fermentation and is used in pre-treatment processes within leather and textile industries. Lactic acid can be easily and cheaply purified using various chemical methods (Vijayakumar et al., 2008).
Using D-lactate dehydrogenase (D-LDH) and L-lactate dehydrogenase (L-LDH) we will be able to generate a metabolic circuit (Figure 3) which should directly convert purified lactate into ethanol with high yields.

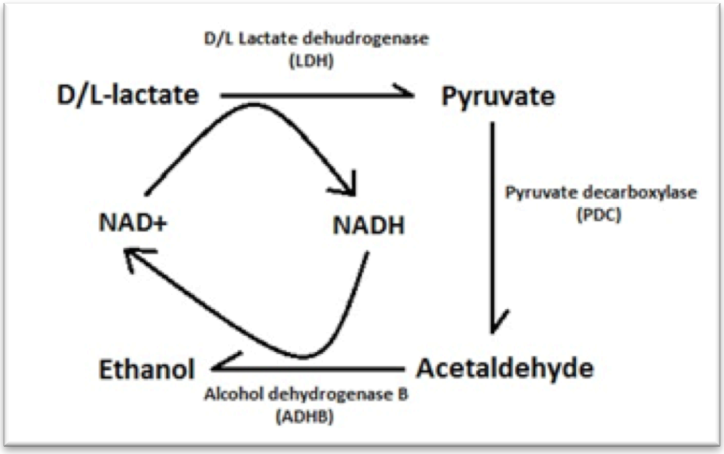


**Figure 3.** Direct conversion of lactic acid to ethanol using LDH and our fusion protein. Note that NADH cofactor is recycled over the course of reaction what directionates the reversible steps catalysed by LDH and ADHB.

## d. Aggregation by biofilms

*Bacillus subtilis* naturally forms biofilms composed of exopolysaccharides and the protein TasA. Production of both of these components is repressed by the transcription factor SinR. It represses the *eps* (exopolysaccharide) and *yqxM* (includes TasA) operons by binding DNA at their regulatory elements. The *eps* and *yqxM* operons contain various genes encoding biofilm formation enzymes and regulatory elements. The repressive functionality of SinR is disrupted by SinI, which binds to SinR disables its' ability to attach to DNA (Whole pathway illustrated in Figure 4a) (Winkelman *et al*, 2009). The upstream regulation of SinI and SinR is very complex and the details are not important for this project.

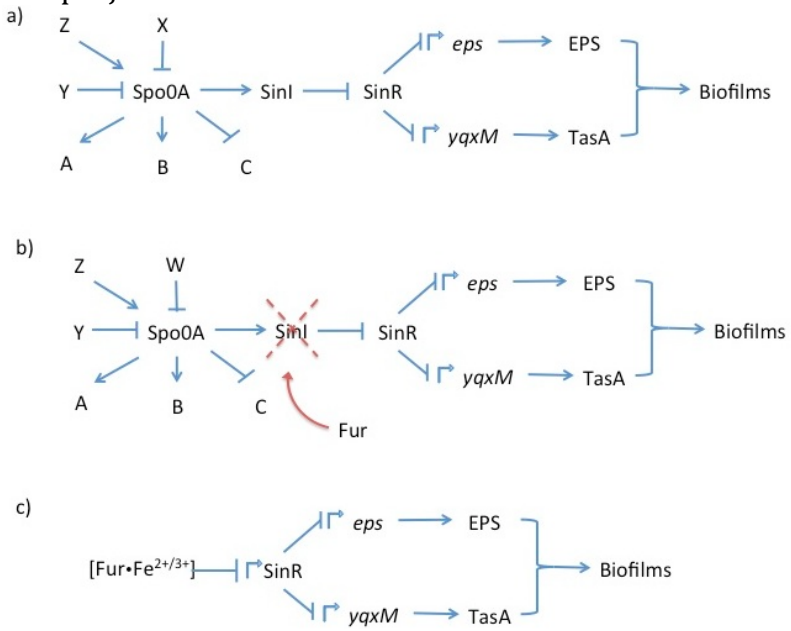


**Figure 4.** Schematic showing the most crucial regulatory sequence elements from the *B. subtilis* master regulator Spo0A, which integrates various cellular signals, to operons and their gene products that create biofilms (a). The genetic modifications we intend to perform in our aggregation subproject (b)&(c) (Winkelman *et al*, 2009).

Upon forming a biofilm, the cells aggregate into a floating pellicle if grown in liquid. In our project, we want to use this natural *B. subtilis* feature as a method of extraction. Furthermore, we intend to make biofilm formation responsive to iron ion concentration in the environment recognisable by the Fur transcription factor. We will attempt to accomplish this by replacing the SinI gene, which is directly downstream of SinR, and normal SinR regulatory elements by a constitutive promoter with a Fur binding box. This would cause biofilm formation derepression in the presence of iron (Figure 4b & 4c).

## e. GenBrick Assembly

GenBrick is based on the proprietary Genabler technology developed for Scottish Enterprise by Ginkgo Bioworks. GenBrick parts are fully BioBrick compatible and can be assembled by normal BioBrick assembly, but they can also be assembled using Genabler assembly, which allows rapid 'single pot' assembly of up to ten parts in a single reaction. Like Golden Gate assembly and its derivatives, Genabler assembly relies on the use of Type IIS restriction enzymes (in this case, EarI), which cut outside of their own recognition sequence to generate user-defined sticky ends. However, in the case of Genabler assembly, these ends are not ligated together directly, but are used to attach linkers with single stranded regions (www.genabler.com) For GenBrick, we wanted to ensure that all parts were RFC10 compatible. We also wanted to facilitate the use of different ribosome binding sites, N and C terminal tags, generation of fusion proteins, etc. We have therefore modified the original Genabler concept to consider two different types of component - 'Bricks' and 'Linkers'. Each assembly consists of alternating bricks and linkers (Figure 5). A copy of the protocol is included in the appendix.

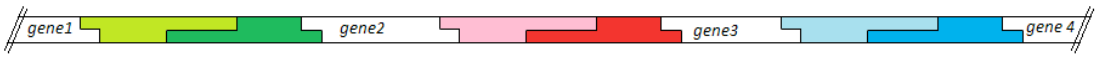


**Figure 5.** Example of GenBrick Assembly
Legend: Genes (bricks) are assembled together with GenBrick. Each brick is flanked by an 'Eye' and a 'hook' linker. All components more than 80 or so base pairs in length are made as Bricks; shorter components are made as Linkers. A typical Brick might be either part of a coding sequence, or a promoter sequence, origin of replication, etc. For the kind of assemblies we are interested in, the most important Bricks are Coding Bricks, which each encode a protein domain.

## f. Modelling

### Host-circuit interaction

Synthetic biologists often design genetic circuitry in isolation, taking little consideration of the host cells in which these circuits will operate. They tend to create specific, local models, which don't capture the circuits' interactions with other host components. This is an oversimplification because the circuit genes and products interact with the host cell in various ways:

- The circuit is dependent upon the resources and machinery available to the cell – so if resources are scarce, this is likely to hinder the circuit transcription and translation.
- The cell needs to replicate, translate and transcribe the additional genes inserted into it and this draws upon the host's resources, which could otherwise be used for metabolism and growth. As a result, if the circuit is long or the genes on it are overexpressed, this can slow down the growth of the host cell.
- The circuit might interact with the cell metabolism in an undesirable manner. For example, its gene products might be toxic to the host. Alternatively, some of the host's metabolic enzymes might inhibit the circuit's production rate; an unwanted side effect.

Failing to take account of those interactions and their consequences at the design stage can cause designs to fail or be sub-optimal.

**Whole-cell model**

With this in mind, we decided to introduce the concept of whole-cell modelling to iGEM: modelling the entire cell and capturing key factors of its life cycle and metabolism. A very abstract, high-level cell "template" could be made thus, or instead a very detailed, richly-informative model, depending on the data available and on the specific application. We can then insert specific circuit models into this whole-cell model and see how the circuit would operate in the context of the cell. In this way, we can create better-informed designs, which have a symbiotic rather than a parasitic relationship with their host.

It would be even better to have a living breathing computer cell that is accessible to everyone, despite its turbid programmatic depths. The way to achieve this would be to have a universal simulation platform with a modular nature, in which different modules can be easily added and removed. We can have the whole cell model running on this platform, and we can easily add any specific circuit models as black-box modules.

This idea can be extended to make the whole-cell model itself into a module, which can run in the simulation platform. Thus it would be possible to choose a whole cell from a library of models, or to program your own one. It may also be possible to turn on/off some features of the whole cell model, customizing it to be more coarse-grained or more fine-grained according to your preferences.

With this ambitious view in mind, we set off to explore this idea and make a first few steps towards implementing it.

# 3. Results

- We tested the growth of our experimental strain, *Bacillus subtilis 168*, under various conditions relevant to the work we are going to perform.
- We tested the Kanamycin resistance conferred by pTG262 in *B. subtilis* and *E. coli*.
- We created an assembly to demonstrate that Fur box is able to repress gene expression upon exposure to high iron concentrations.
- We cloned out and characterised a Fur transcription factor.
- We cloned a Neisserial Ferric binding protein A (FbpA) as a BioBrick.
- We generated a fusion of Pyruvate decarboxylase (Pdc) and Alcohol dehydrogense B (AdhB). We characterised it thoroughly and demonstrated that such co-localisation can increase ethanol production at least twofold.
- We cloned SinR and analysed biofilm formation in WT *B. subtilis*.
- Modelling results are only available online. Sorry for the discomfort.
- We had a great summer, and plan to have fun at the Regional Jamboree.

## a. Characterization of Bacillus subtilis 168 as a chassis

In order to better understand what conditions are best suitable for growth of our experimental strain, *Bacillus subtilis* 168, we tested the growth under various conditions relevant to the work we would perform later. First we measured the growth curves at various ethanol concentrations to confirm the highest ethanol concentration suitable for cell growth, which was 4%. Afterwards, we measured the growth curves at various concentrations of iron, copper, nickel, cobalt, zinc and manganese.
To test for induced kanamycin resistance, *B.subtilis* 168 and *E. coli* JM109 were each transformed with the vector pTG262 (BBa_I742123) with an RFP biobrick insert cassette. The number and size of colonies, which grew on each of the agar plates, were recorded.

## b. Fur Box Assembly and Fur transcription factor Assay

Using the fur box assembly two transformations were made and plated on IPTG + chloramphenicol plates and left overnight. In one plate, 4 out of 9 colonies were fluorescent; the other five had religated and were red. The other plate had a success rate of 8 out of 20 (the competent cells were concentrated x10 before plating). Figure 6 shows the assembly fluorescing under UV when no iron was present and figure 7 shows the gel of restriction-digestion using PstI and XbaI.

**Figure 6. Fur box Assembly**

Legend: after transforming competent cells with the assembly and leaving overnight on an IPTG + chloramphenicol plate, 2 green fluorescent colonies were restreaked. A gel was performed to show the parts had correctly assembled.

**Figure 7. Fur Box Digestion**

Legend: On the left is the ladder, the first column is the undigested assembly which shows only one band (the second lower band is an artifact due to leakage). The second column contains the digested assembly. The size of PLac_LacZ + linkers + GFP

The Fur gene was restricted and ligated into pSB1C3 using XbaI and PstI. Once that was done, PLac_LacZ was placed upstream of the Fur gene to over-express it. It was assumed that over-expression of Fur could increase bacterial tolerance to increased iron concentration due to enhanced perception of environmental iron. This was analysed experimentally by culturing cells induced and un-induced to express Fur in LB, supplemented with various iron concentrations (Figure 8).

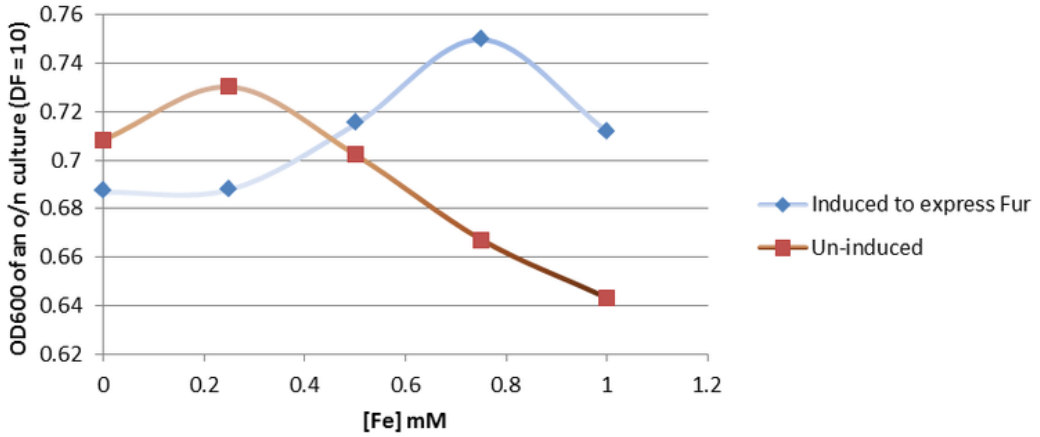


**Figure 8.** Results obtained indicate that indeed the cells that contain more copies of Fur protein survive better in medium with increased iron levels.

## c. Functional analysis of FbpA

The coding sequence of the Neisserial FbpA protein was cloned from a plasmid obtained from within the University of Edinburgh. Two forms of the protein were cloned: the full length native coding sequence FbpA1 BBa_K1122702 and the coding sequence less the putative signal peptide FbpA2 BBa_K1122703. FbpA was expressed using Plac promoter. Overexpression of FbpA in high iron concentration (Figure 9) gave twofold results: Overexpression of FbpA increases tolerance of *E. coli* to iron. As it can be observed on Figure 9 the cells producing the protein can grow closer to the iron source than those that do not (JM109). Upon closer inspection one can observe that cells overexpressing FbpA have slight red pigmentation. This could indicate that cells accumulate iron.

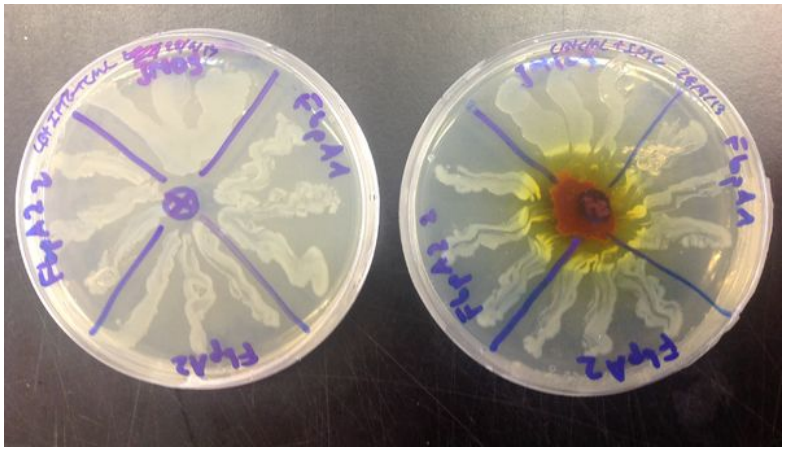

**Figure 9.** Ferric citrate was placed on the middle of the right plate. Control cells plated on no additional iron conditions (left), cells overexpressing FbpA (plate parts labelled FbpA1, FbpA2 and FbpA22) in high iron conditions (right). Control cells without FbpA are labelled JM109.

## d. pET fusion and bioethanol

In order to generate a fusion of Pyruvate decarboxylase (Pdc) and Alcohol dehydrogense B (AdhB), Mutagenesis with Blunt- Ended Ligation (MABEL) was used. A pair of primers were designed complementary with 3' end of Pdc and 5' end of AdhB. Following a PCR a product was generated which lacked STOP codon of Pdc, RBS and a start codon of AdhB.
Following blunt-ended ligation presence of gene fusion was conirmed via sequencing. Presence of fused protein was presented by SDS-PAGE (Figure 10) following addpition of Plac upstream of fused and non-fused enzymes.

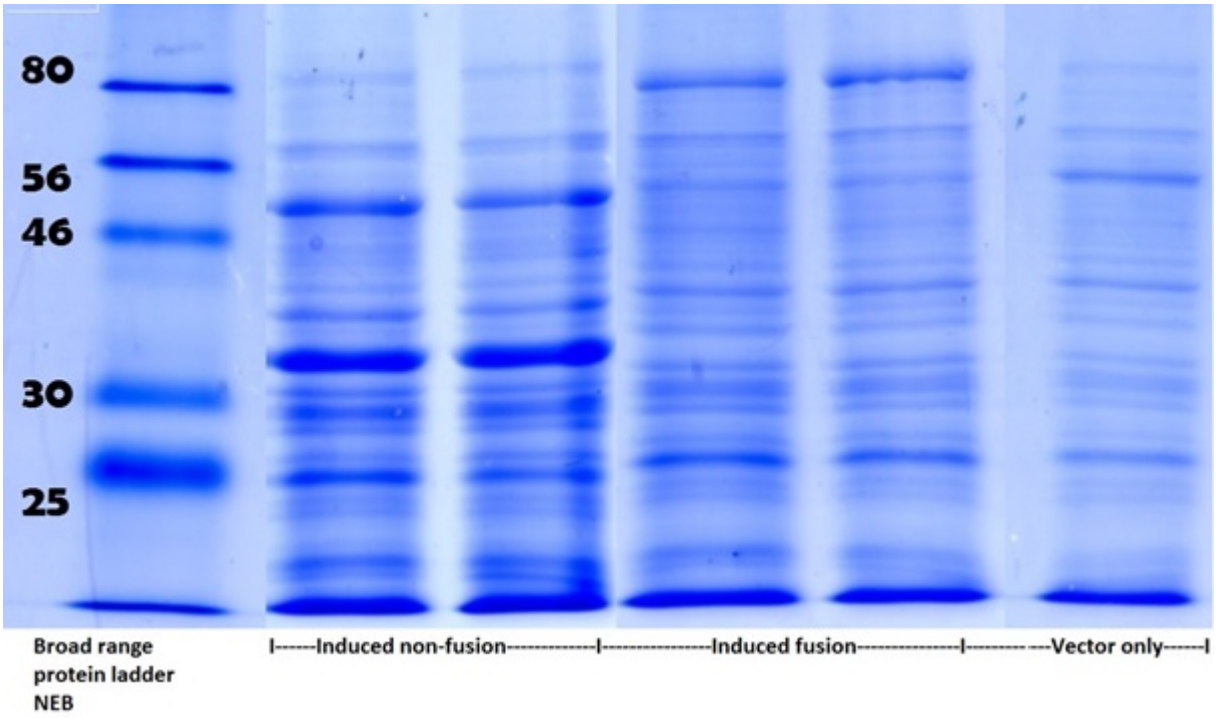


**Figure 10.** This shows the SDS-PAGE following Coomassie Brilliant Blue G250 staining. Within the induced non-fusion lanes, two intense bands are present (with size corresponding to pdc and adhB). Within the induced fusion lanes, the previously described bands are missing but an additional band of increased size is observed. In the vector only lane, none of above described bands are present.

It was established that the highest ethanol yields could be achieved in glucose as a carbon source. LB+ 4% glucose was used for ethanol production. Figure 11 presents ethanol levels achieved in the experiment. Great increase in alcohol production was observed in fusion enzyme state.

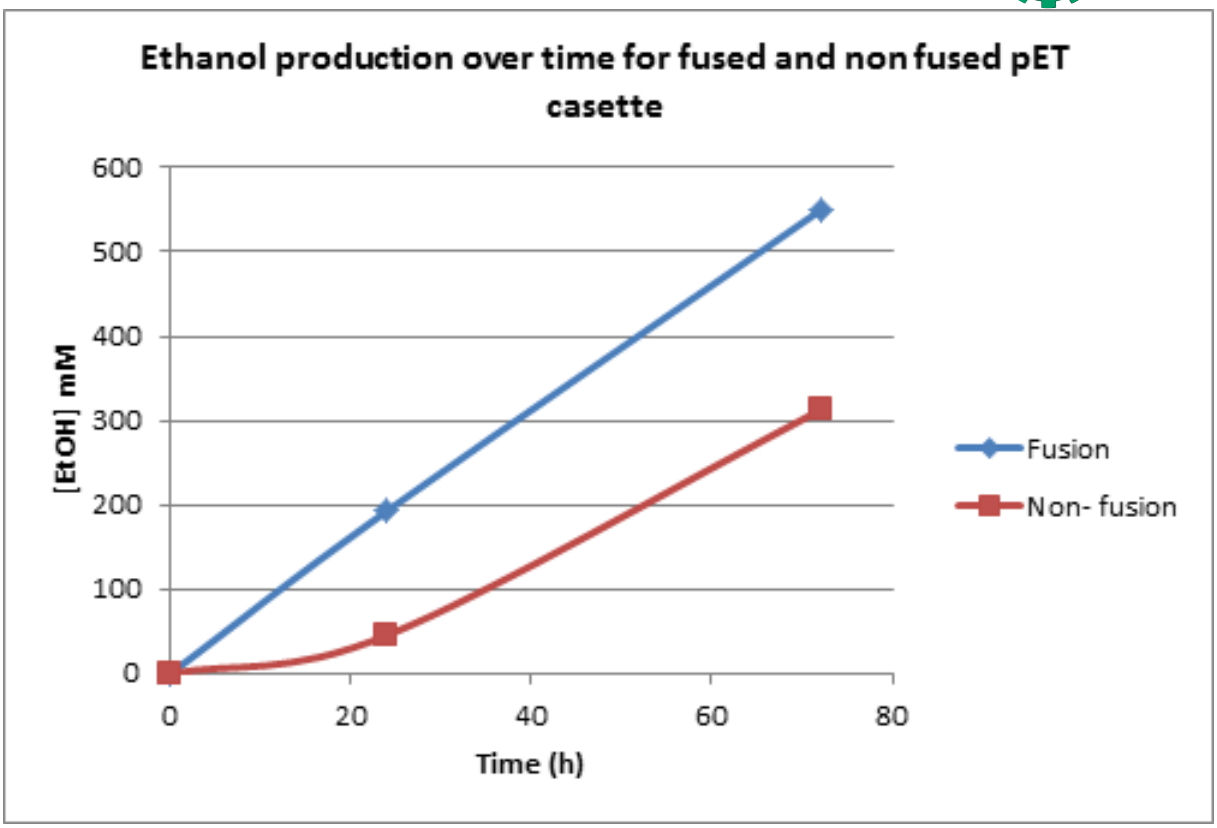


**Figure 11.** This is an ethanol production graph. The cultures were first incubated for 24 hr in an aerobic environment. Following that, they were switched into fermentative conditions for 48 hr.

## e. Human Practices

In an industry such as waste treatment, there is no doubt of the global applications. Almost every country has some kind of factory or waste producing business that could benefit from treatment. In order to reach that global environment, we must start at the local level. In Scotland, as in other countries, there are already certain chemical treatments in place that may precipitate the contaminant, but these can be expensive and do not do anything special with the waste. What we aim to do is not just remove this contaminant, but also use it to make a useful product.

Our system would fit like a puzzle piece into the current industrial settings as an add-on to an existing factory. In Scotland, there are three main industries that could be easily targeted with our waste treatment: textiles, leather, and whisky. While investigating these particular businesses, we visited a whisky distillery to see how our treatment option might fit in, about which you can find more information online.

As the vote on Scottish independence grows nearer, a closer look at the proposed economy is essential. An ever-expanding industry in Scotland is the life sciences, which, unlike the Aberdeen oil industry, will continue to develop as new innovations are made. This is based on the premise that the life sciences are not tied to a shrinking supply of some element or compound, but are limited only by imagination and funding. The creation of this system would further move to promote and develop the study life sciences because of its potential for growth; both locally and globally.

We are all very happy to have become members of an iGEM team, but many bright people around the world and even in Edinburgh are unaware of this amazing competition or of Synthetic Biology and Genetic Engineering at all. This is why we looked for as many opportunities to present these concepts to the target audience of other well-educated students, from diverse scientific backgrounds and nationalities.

## 4. Conclusions

The ethical implications of our project go far beyond the treatment of waste generated by traditional Scottish industries. We believe that using the system that we intended to build one could remediate various industrial waste cheaply and efficiently. This would be particular important for developing countries where both heavy metal and organic pollution has a heavy burden on the environment.
Considering the scientific aspect of work performed we believe that one of our greatest achievements was introduction of novel BioBrick assembly standard: GenBrick. GenBrick is efficient and reliable. It can be used for generation of fusion proteins or introduction of novel and interesting features in-between assembled genes. Our detailed description of the system enables use of it by iGEM teams to come. Moreover, great increase in ethanol yields achieved using Pdc and AdhB fusion protein was the most spectacular of results obtained.
Finally, the ambitious project of modelling the whole cell provided a ground-work onto which subsequent generations of iGEM'ers will be able to apply their own designs in order to test effect of those on the model organism used.

## 5. Future Work

The research performed in this project and presented on our Wiki provides us with some useful insights and unveils the potential lying in:

1. Bioethanol and fusion proteins

Our work showed that the fusion of Pdc and AdhB was more efficient in the production of ethanol from pyruvate than free proteins. This indicates the potential of using protein fusions for facilitating other metabolic reactions as well. Future work should focus on creating multiple protein fusions to achieve even higher yields of ethanol or other substances. Moreover, this project demonstrated the metabolic stress caused by misbalancing cellular coenzyme NAD+ and NADH levels. In the future, scientists should focus on genetically engineering and overexpressing not only the proteins necessary to achieve the primary product, but also metabolic networks necessary to maintain the cellular coenzyme and cofactor balance.

2. Metal recognition

We investigated the Fur protein and identified its potential benefit in increasing bacterial survival in heavy iron conditions. This work suggests that overexpression of relevant transcription factors increases bacterial survival in environment supplemented with relevant heavy metal ion. What remains to be done is to link the enhanced perception of particular metal to change in the behavior of the cell.

3. Metal binding

We investigated the iron binding activity of Neisserial ferric binding protein, FbpA and added it to the registry. However a vast amount of other metal binding entities exist, such as the siderophores produced by non-ribosomal peptide synthases. Furthermore, protein complexes which produce metal nanoparticles may prove to be more efficient when it comes to large scale metal ion extraction.

4. Aggregation

Much work remains to be done in that area in order to obtain our initial goals. We have successfully cloned genes required for control over biofilm formation and performed qualitative analysis of this process. Assembly of generated parts needs to be done in order to test our initial hypothesis.

# Thank You For Your Attention!

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# 7. Appendices

## 7.1 List of Parts Submitted

| | Type | Description | Designer | Length |
|---|---|---|---|---|
| BBa_K1122001 | Composite | GFP (BBa_E0040) under the control of lac promoter | Lina Gasiūnaitė | 1151 |
| BBa_K1122003 | Composite | mTagBFP (BBa_K592100) under the control of lac promoter | Lina Gasiūnaitė | 1137 |
| BBa_K1122004 | Coding | GFP (BBa_E0040) compatible with Genabler assembly | Lina Gasiūnaitė | 736 |
| BBa_K1122007 | Coding | AmilCP (BBa_K592009) compatible with Genabler assembly | Lina Gasiūnaitė | 685 |
| BBa_K1122008 | Coding | mTagBFP (BBa_K592100) compatible with Genabler assembly | Lina Gasiūnaitė | 721 |
| BBa_K1122009 | Plasmid | pDonor2 | Lina Gasiūnaitė | 2462 |
| BBa_K1122010 | Reporter | Genabler acceptor RFP cassette | Lina Gasiūnaitė | 1034 |
| BBa_K1122011 | Reporter | Genabler acceptor lacZα cassette | Lina Gasiūnaitė | 376 |
| BBa_K1122674 | Translational_Unit | Plac+fused PDC-ADH | Aleksandra Lewicka, Jan Lyczakowski | 3459 |
| BBa_K1122000 | Coding | SinR transcription factor | Dainius Tautvaisas | 333 |
| BBa_K1122005 | Coding | dsRED (BBa_E1010) compatible with Genabler assembly | Lina Gasiūnaitė | 697 |
| BBa_K1122006 | Coding | mCherry (BBa_J06504) compatible with Genabler assembly | Lina Gasiūnaitė | 730 |
| BBa_K1122069 | Regulatory | Ferric uptake repressor box | Hugo Villanueva | 15 |
| BBa_K1122222 | Regulatory | pSpac - LacZ | Aleksandra Lewicka | 390 |
| BBa_K1122666 | Coding | Ferric uptake repressor | Hugo Villanueva | 450 |
| BBa_K1122667 | Coding | Enterobactin synthase component F | Kyle Rothschild-Mancinelli | 4020 |
| BBa_K1122668 | Coding | Enterobactin synthase component D | Kyle Rothschild-Mancinelli | 630 |
| BBa_K1122669 | Coding | Enterobactin synthase component E | Kyle Rothschild-Mancinelli | 1611 |
| BBa_K1122670 | Coding | Enterobactin synthase component B | Kyle Rothschild-Mancinelli | 858 |
| BBa_K1122671 | Coding | Enterobactin synthase component S | Kyle Rothschild-Mancinelli | 1251 |
| BBa_K1122672 | Coding | Alcohol dehydrogenase E (AdhE) | Aleksandra Lewicka, Jan Lyczakowski | 2589 |
| BBa_K1122673 | Coding | Ethanol production module | Aleksandra Lewicka, Jan Lyczakowski | 3014 |
| BBa_K1122675 | Other | SinI Upstream Flank | Kyle Rothschild-Mancinelli | 999 |
| BBa_K1122676 | Translational_Unit | IPTG inducible pdc and adhB | Aleksandra Lewicka, Jan Lyczakowski | 3660 |
| BBa_K1122678 | Translational_Unit | pSpac - LacZ - pdc- adhB | Aleksandra Lewicka, Jan Lyczakowski | 3450 |
| BBa_K1122679 | Translational_Unit | pSpac - LacZ - fused pdc and adhB | Aleksandra Lewicka, Jan Lyczakowski | 3412 |
| BBa_K1122680 | Translational_Unit | pLac - LacZ - Fur | Aleksandra Lewicka, Jan Lyczakowski | 1056 |
| BBa_K1122681 | Translational_Unit | pSpac - LacZ - Fur | Aleksandra Lewicka, Jan Lyczakowski | 846 |
| BBa_K1122682 | Other | SinR Downstream Flank | Kyle Rothschild-Mancinelli | 999 |
| BBa_K1122683 | Regulatory | Plac_LacZ_RBS (B0030) | Aleksandra Lewicka | 623 |
| BBa_K1122684 | Regulatory | Plac_LacZ_RBS (B0032) | Aleksandra Lewicka | 621 |
| BBa_K1122685 | Regulatory | Plac_LacZ_RBS (B0033) | Aleksandra Lewicka | 619 |
| BBa_K1122700 | Coding | L lactate dehydrogenase (L-LDH) | Jan Lyczakowski | 993 |
| BBa_K1122701 | Coding | D lactate dehydrogenase (D-LDH) | Jan Lyczakowski | 1002 |
| BBa_K1122702 | Coding | Ferric ion-binding protein (FbpA) | Harry Thornton | 999 |
| BBa_K1122703 | Coding | Ferric ion-binding protein (FbpA) without a signal peptide | Harry Thornton | 936 |

7.2 GenBrick Assembly Protocol

# Design and Preparation - Linker and Segment

## 1. Overview

- Utilise the linker designer software from the EdiGEM 2013 wiki to design Eye and Hook linker oligo pairs
- Linker and Segment oligos can be custom-made as single-stranded, unphosphorylated DNA
- Forward and reverse oligo pairs are mixed and phosphorylated prior to annealing

## 2. Preparation

- Re-suspend oligo in nuclease-free water to 100 µM (as per instructions)
- Phosphorylation reaction (not required if oligos with 5' phosphate are ordered)

| component | volume (µL) |
|---|---|
| 10X T4 Polynucleotide Kinase buffer | 5 |
| T4 Polynucleotide Kinase (NEB M0201) | 0.5 |
| 10 mM ATP | 5 |
| Forward oligo | 20 |
| Reverse oligo | 20 |
| 37°C/30 min | |
| 5 M NaCl | 0.5 |
| 95°C to RT | |

- Annealing is achieved by addition of 5 µL 5 M NaCl (50 mM [final]) prior to heat denaturation at ≤ 95°C and slow cooling to Room temperature.

# Pre-assembly

## 1. Eye-Part-Hook Preparation

**a) Using 3-part pathway as example:**

- Acceptor Vector cassette (+ promoter); Acc_RFP
- LacZ truncated gene (+ PLac); PLac_LacZ
- Green fluorescent protein; GFP

**b) Digestion-Ligation reaction of Eye-Part-Hook (E-P-H) (Overnight)**

- Acc_RFP-E + Acc_RFP-P + GFP-H
- PLac_LacZ-E + PLac_LacZ-P + Acc_RFP-H
- GFP-E + GFP-P + PLac_LacZ-H

| component | volume (µL) |
|---|---|
| 50 nM Part plasmid DNA | 10 |
| 500 nM eye oligo pair | 10 |
| 500 nM hook oligo pair | 10 |
| 10 mM ATP | 5 |
| 10X NEBuffer 4 | 5 |
| EarI (NEB R0528) | 1 |
| T4 DNA Ligase (NEB M0202) | 1 |
| Sterile water | 8 |

| step | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| reaction | digestion | ligation | digestion | ligation | digestion | ligation | digestic |
| temp | 37°C | 16°C | 37°C | 16°C | 37°C | 16°C | 37°C |
| time | 90 min | 30 min | 30 min | 15 min | 15 min | 15 min | 15 mir |

**c) Purification of Digestion-Ligation E-P-H product to remove non-ligated DNA/plasmid**

- Run 50 µL E-P-H Digestion-Ligation reaction on 1% agarose gel
- Or: QIAquick PCR Purification kit can be used if Part plasmid does not carry resistance/marker

## Assembly

### 1. Incubation

- Use 5 µL each E-P-H required for assembly
- Add x µl 10X NEBuffer 4 to make 1X final concentration
- Incubate 30-60 minutes at Room Temperature
- 3-part Example:
  - 5 µL Acc_RFP E-P-H ([Acc_RFP -E]+[ Acc_RFP -P]+[GFP-H])
  - 5 µL PLac_LacZ E-P-H ([PLac_LacZ-E]+[PLac_LacZ-P]+[Acc_RFP-H)
  - 5 µL GFP E-P-H ([GFP-E]+[GFP-P]+[PLac_LacZ-H])
  - 2 µl NEBuffer 4
  - 3 µL sterile water
- If larger number of E-P-H in assembly, adjust 10X NEBuffer 4 and water accordingly

### 2. Transformation

- Use 10 µL assembly mix to transform 50 µL NEB 10-beta competent cells C3019H
- Culture above assembly example on LB agar plates with chloramphenicol and IPTG
- Incubate overnight at 37°C (further growth at RT if colonies require)