Production of industrially relevant proteins using cell-wall deficient cells

A Thesis

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Indian Institute of Science Education and Research Pune in partial fulfillment of the requirements for the BS-MS Dual Degree Programme

by

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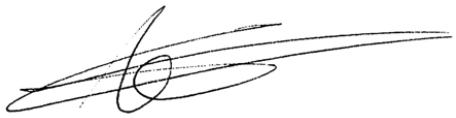
Certificate

This is to certify that this dissertation entitled ‘Production of industrially relevant proteins using cell-wall deficient cells’ towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune, represents study/work carried out by Chinmay Patwardhan at Leiden University under the supervision of Dennis Claessen, Professor Microbiology and Director of Education, Institute of Biology, and Marjolein Crooijmans, Ph.D. candidate, Institute of Biology, during the academic year 2022-2023.

Name and Signature of Supervisor

D. Claessen



Date

21/04/2023

This thesis is dedicated to my parents.

Declaration

I hereby declare that the matter embodied in the report entitled ‘Production of industrially relevant proteins using cell-wall deficient cells’ are the results of the work carried out by me at the Department of Microbial Biotechnology and Health, Institute of Biology, Leiden University under the supervision of Dennis Claessen, Professor Microbiology and Director of Education, and Marjolein Crooijmans, Ph.D. candidate, and the same has not been submitted elsewhere for any other degree.

Chinmay Patwardhan

24 March 2023

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Abstract

Over the last few decades, *E. coli* cells have been increasingly used in industrial biotechnology to produce products of high economic value. This includes enzymes, therapeutics, and dietary supplements. However, currently used *E. coli* systems have drawbacks, making them sub-optimal for producing certain types of products. L-form *E. coli* could target these issues and be established as an industrially viable strain for specific commercial products. L-forms are cell wall deficient strains of bacteria that can be created using mutations, spacial constraints, or antibiotic treatments. Their property of L-form switching could provide robustness in industrial settings. This project deals with understanding the properties of an *E. coli* L-form strain and how it responds to standard DNA manipulation and transformation techniques. The project also looks at this strain's ability to produce simple fluorescent proteins and goes on to scale up to more complex proteins like enzymes. Using two enzymes, α-amylase and β-galactosidase, we show that the L-form strain can also produce functional enzymes. We were able to come up with a new protocol for reliably transforming L-form *E. coli* and were also able to determine the optimal conditions for protein expression on solid media. Scaling up experimentation with bioreactors and testing the enzymes in a liquid assay format would allow for a better understanding of L-form *E. coli*. Creating a stable L-form from an industrial strain would also aid in comparing protein production between various strains.

Acknowledgments

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Contributions

Contributor name	**Contributor role**
Chinmay Patwardhan, Marjolein Crooijmans, Dennis Claessen	Conceptualization Ideas
Chinmay Patwardhan, Marjolein Crooijmans, Dennis Claessen	Methodology
Chinmay Patwardhan, Marjolein Crooijmans	Software
Chinmay Patwardhan, Marjolein Crooijmans, Dennis Claessen	Validation
Chinmay Patwardhan, Marjolein Crooijmans, Dennis Claessen	Formal analysis
Chinmay Patwardhan, Marjolein Crooijmans, Dennis Claessen	Investigation
Chinmay Patwardhan, Dennis Claessen, Marjolein Crooijmans, All ClaessenLab members, Prof. Erik Vijgenboom, Prof. Lennart Schada von Borzyskowski, Bas van Woudenberg, Mark Arentshorst, Mandy Hulst, Jonas Hermant, JR Quant, Ben Nannings, Davy de Witt	Resources
Chinmay Patwardhan, Marjolein Crooijmans	Data Curation
Chinmay Patwardhan	Writing - original draft preparation
Chinmay Patwardhan, Marjolein Crooijmans	Writing - review and editing
Chinmay Patwardhan, Marjolein Crooijmans	Visualization
Marjolein Crooijmans, Dennis Claessen	Supervision
Dennis Claessen	Project administration
Dennis Claessen	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy.

Chapter 1: Introduction

Bacteria are single-celled, metabolically-active microorganisms that lack a nuclear membrane. While they may appear relatively simple, they're quite sophisticated and highly adaptable. They are ubiquitous and can be remarkably adaptable to changing environments by natural selection. That's why their importance in biotechnology and medicine cannot be overstated.

Along with different kinds of yeasts and molds, bacteria have been previously used in the agricultural, pharmaceutical, and chemical industries. 'Industrial microbiology' refers to the branch of biotechnology that uses microorganisms and microbial cell factories to create industrial products in mass quantities. Organisms like *Escherichia coli*, *Saccharomyces cerevisiae,* and *Bacillus subtilis* and their various strains are widely used to produce various industrially relevant products like human hormones, enzymes, therapeutics, vaccines, supplements, etc. (Walsh, 2018)

However, there are several problems and concerns associated with these production systems, making them sub-optimal for producing certain biological products. Current bacterial systems have low protein expression levels, slow growth, and poor robustness (Yang et al., 2018). *E. coli* has several cytoplasmic endogenous proteases that can cause proteolytic degradation, and this is a drawback for *E. coli* as a production system. However, in standard producer strains, this problem has been avoided by either using protease-deficient strains or the secretion of protein in the periplasm, where there are fewer proteases (Batra & Rathore, 2016). Due to the presence of a cell wall, the periplasmic expression of proteins is limited since there is limited periplasmic space. Production of some large proteins like full-length antibodies continues to be a challenge due to low productivity in *E. coli* (Lee & Jeong, 2015). Due to its reductive cytoplasmic environment, the formation of disulfide bonds is seriously affected(Spadiut et al., 2014). Additionally, cytoplasmic production in *E. col*i allows high intracellular product yields, but it is often associated with inclusion body formation (Khodabakhsh et al., 2013). Inclusion bodies are aggregates of proteins within the cell, which lead to a reduction in their activity. This is also a prominent issue with over-expressing proteins in normal bacterial systems since it results in protein misfolding and aggregation.

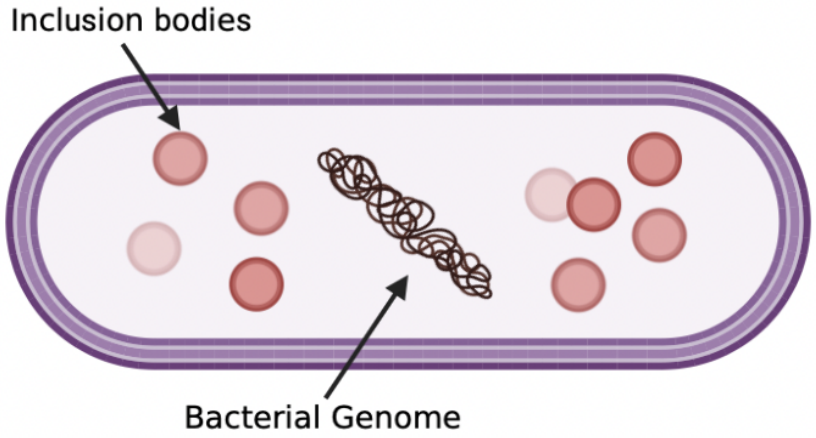


Figure 1: Inclusion bodies in gram-negative bacteria formed due to protein aggregation and misfolding. Created using Biorender.

These problems could potentially be tackled with the use of L-form, cell-wall deficient (CWD) bacteria. They are named after the Lister Institute, where they were discovered in 1935 by Emily Kleineberger (Klieneberger, 1935). Since then, L-forms have come a long way. Essentially, L-forms are CWD strains of bacteria that can be differentiated as either spheroplasts or protoplasts, depending on whether they originate from Gram-negative or Gram-positive bacteria, respectively (Nishida, 2020). L-forms can further be classified as 'unstable' or 'stable' depending on whether or not they can revert to their parent form (with a cell wall) (Allan et al., 2009). Usually, unstable forms need some sort of cell-wall-deficiency inducers (like beta-lactam antibiotics) in the growth medium to remain in the L-form (Allan et al., 2009; Gumpert & Hoischen, 1998; Kawai et al., 2018).

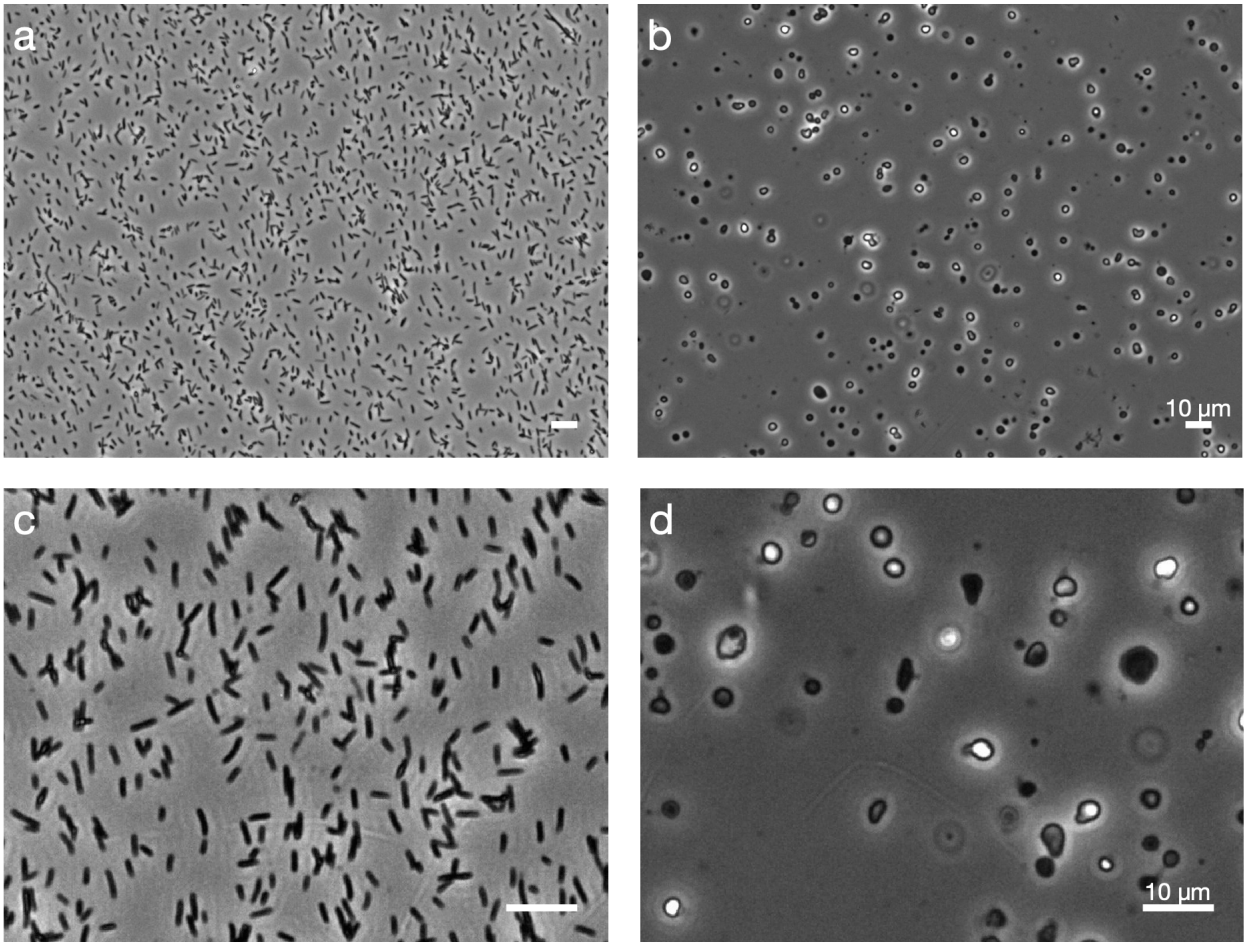


Figure 2: Comparison of walled and L-form *E. coli* cells. (a,c) *E. coli* DH5a strain at 20x and 40x magnification, respectively. (b,d) L-forms of K-12 MG1655 *E. coli* strain at 20x and 40x magnification, respectively. Clear spherical morphology was observed in the L-form cells

The purpose of this project is to explore the industrial applications of L-form strains of *E. coli* bacteria. We want to test the efficiency of *E. coli L-forms* as a new chassis for recombinant protein production, specifically, those proteins which are currently difficult to express in normal *E. coli* or whose yields might be sub-par at an industrial level. These proteins include enzymes and antibody fragments. Theoretically, the L-forms of *E. coli* should be able to express any proteins and secrete them with the addition of an appropriate signal peptide. However, data for this is not currently available (Hoischen et al., 2001). Current knowledge of *E. coli* L-forms lacks the understanding of how much of the outer membrane and cell wall is intact. This could play a significant role in understanding how we produce proteins in the *E. coli* L-form strain.

Due to their unique properties of surviving and dividing without a cell wall, L-forms of bacteria might show advantages over conventionally used bacterial systems for

recombinant protein expression. Various types of protein products, like enzymes, enzyme activators, and antibodies, have been synthesized using L-form expression systems in the past (Gumpert et al., 1996; Gumpert & Hoischen, 1998; Hoischen et al., 2001; Kujau et al., 1998; Matsuda et al., 2014; Osawa & Erickson, n.d.; Rippmann et al., 1998; Yang et al., 2018, 2019). L-forms and CWD strains have also been shown to be stable in industrial fermenters. L-forms increase their surface areas and thus increase their membrane production compared to walled cells. Improving the yield of some hydrophobic commercial products should be possible by expressing them in L-forms. Protein secretion might also be facilitated through this effect with respect to the increased surface area over which secretion can take place (Errington et al., 2016).

Bacteria, like all prokaryotes, need to transport proteins across their membranes for secretion, survival, or virulence. They achieve this through the use of dedicated protein secretion systems (Green & Mecsas, 2016). Gram-negative bacteria sometimes utilize a two-step Sec- (General Secretion) or Tat- (Twin Arginine Translocation) dependent pathway to secrete proteins. Proteins are able to cross the inner membrane with the help of the Sec or Tat pathway and then utilize a Type II or Type V system to cross the outer membrane. However, since L-form bacteria lack a cell wall, the presence and functionality of these Type II or Type V systems is unknown (Yang et al., 2018, 2019).

Signal peptides are sequences of proteins that are recognized by secretion systems. Each secretion system can recognize specific signal peptides and hence certain proteins can only be secreted by certain systems. The Sec system can recognize a few signal sequences, and some of them have been introduced into inducible plasmid vectors to make it easier to secrete recombinant proteins. The Sec Pathway translocates proteins across the cellular membrane in an unfolded state and consists of three parts: a protein targeting component, a motor protein, and a membrane-integrated conducting channel. After secretion, the signal sequence gets cleaved off, and the protein is able to fold properly outside the cell.

On the other hand, the Tat system usually secretes proteins that are already folded (Green & Mecsas, 2016). This system is important because not all proteins can be folded properly outside the cell. Some proteins that require specific conditions to fold, like post-translational modifications or chaperone proteins, must be folded completely inside the cell before secretion to ensure proper functionality.

Using different signal peptides to target the two main secretion systems (Sec and Tat) could be an approach to enhancing protein expression yield (Freudl, 2018; Gumpert et al., 1996; Gumpert & Hoischen, 1998; Kujau et al., 1998; Rippmann et al., 1998; Zhou et al., 2016). Since the cell wall is ambiguously present in L-forms, this would alter the

secretion systems which are available for use. Looking closely at the Sec and Tat pathways (Figure 3), they would still be functional in the absence of the outer membrane as their subunits are only present on the inner membrane.

One major advantage of L-forms over walled strains is that protein products can be secreted directly into the extracellular space with the help of signal peptides attached to the product. In walled cells, the products are secreted into the periplasm, where the space is limited (Allan et al., 2009). This results in non-secreted proteins being concentrated in the cytoplasm as the periplasmic space gets saturated. This strongly increases the risk of the formation of inclusion bodies for the expression of mini antibodies (Kujau et al., 1998), scFv antibodies (Rippmann et al., 1998) and other recombinant proteins (Gumpert & Hoischen, 1998).

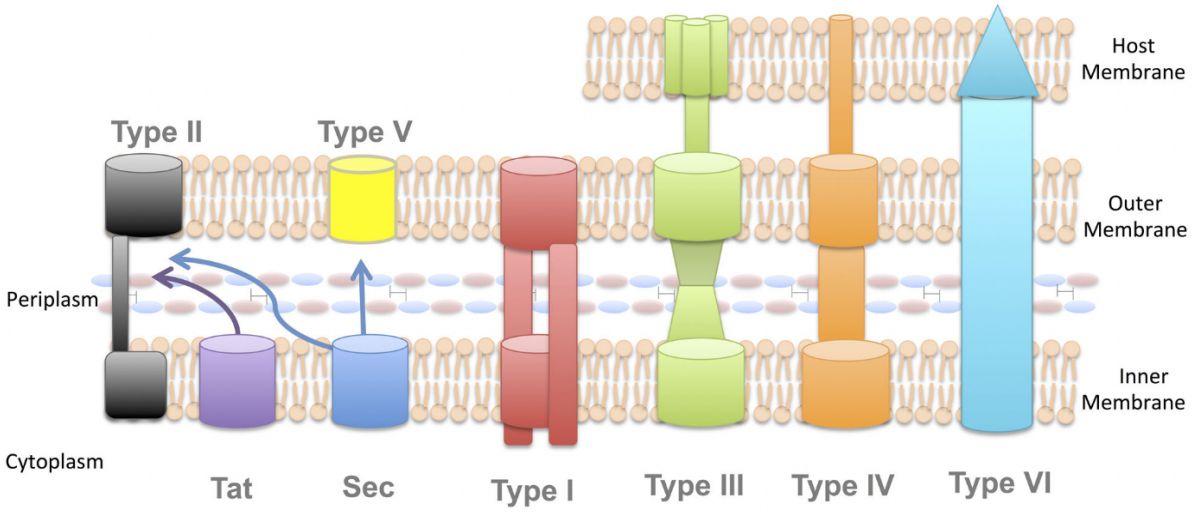


Figure 3: Secretion Systems in Gram Negative bacteria. The Sec Pathway translocates proteins across the cellular membrane in an unfolded state, while the Tat Pathway usually secretes proteins that are already folded. Reproduced from (Green & Mecsas, 2016)

There are well-documented ways of generating Stable L-forms using a standardized four-step method of Induction, Selection, Stabilisation, and Adaptation (Allan et al., 2009). Typically horse serum is often used as an additional supplement as it improves the quality of growth and is often necessary for the growth of protoplast L-form strains on agar media (Allan et al., 2009). Other ways of generating L-forms can include gene mutations that contribute to peptidoglycan synthesis. L forms can also be generated without cell wall-inhibiting substances or genetic mutations/switches. This can be

achieved using spatial constraints. Microfluidic devices can be built that limit peptidoglycan synthesis by extreme spatial confinement. Using a constricted biological corridor, connecting adjacent microhabitats, L-form bacteria can be derived. This uses a microfluidics-based "adaptive ecosystem", which results in the formation of shape-shifting phenotypes similar to L-forms (Männik et al., 2009).

A stable strain of L-form *E. coli* has been created in the Claessen Lab at the University of Leiden from the parent strain *Escherichia coli* K12 MG1655 (unpublished data). This strain has a fluorescent *gfp* gene integrated into its genome and is resistant to beta-lactam antibiotics like penicillin. Officially labeled as *Escherichia coli* K12 MG1655 *gfp*$^{+}$ *PenG*^{R} (L-form), the rest of this thesis refers to it as *E. coli* L-forms (Supplementary Table 1: Bacterial Strains).

In this project, we wanted to study the properties of this *E. coli* L-form strain and evaluate its feasibility of being made into an industrially viable protein-producer strain. The project is divided into three main parts. The first part looks at the properties of the *E. coli* L-form strain. The second part of the project builds on top of the understanding derived from the first part and assesses the strain's potential to produce simple proteins. The third part finally looks at the strain's capabilities to produce functional proteins like enzymes and compares its abilities to standard industrial strains like *Escherichia coli* BL21 DE3.

L-forms of bacteria are naturally competent and capable of taking up extracellular DNA in various ways (Kapteijn et al., 2022). However, due to the lack of a cell wall, the L-forms of *E. coli* are also fragile. Unlike walled strains, they can't survive the stress of being frozen and defrosted at -80°C as a glycerol stock or a competent cell stock (unpublished data). So, there aren't any standard protocols for DNA manipulation and transformation for these L-form *E. coli* strains. That is the starting point for the first part of the project. We want to look at the properties of this *E. coli* L-form and how the strain reacts to standard methods for DNA manipulation and transformation. This part aims to make it possible to manipulate this strain for future experiments reliably.

The second part of the project looks at the L-form strain's ability to produce simple proteins. We chose two fluorescent proteins, mCherry and tdTomato, which vary in size but are fairly standard markers used in industrial microbiology. This part of the project also aims to determine the optimal environmental conditions for the *E. coli* L-form strain to produce proteins. It also goes deeper into the understanding of a unique phenomenon observed in L-form bacterial cells, called L-form switching (Kawai et al., 2018; Mickiewicz et al., 2019; Petrovic Fabijan et al., 2022). It is where wall-deficient cells are able to transition into walled forms in the absence of beta-lactam antibiotics

and osmoprotective media (Figure 4: L-form switching). This property of L-forms makes it possible to store them at -80°C as a glycerol stock after they have switched to their walled form. The results of this part of the project are essential to designing the third part of the project.

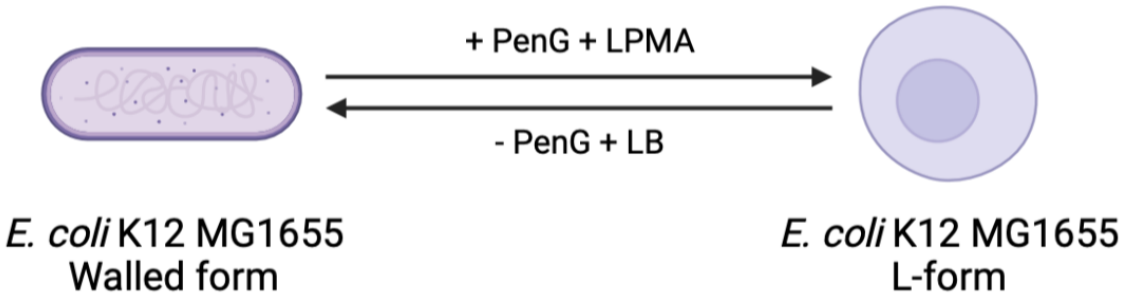


Figure 4: L-form switching. Cells are able to switch between walled and wall-deficient forms depending on environmental conditions, especially the osmoprotective media and beta-lactam antibiotics like penicillin. Created using Biorender.

The third part of the project aims at studying whether *E. coli L-forms* could produce more complex proteins like enzymes and whether those enzymes were catalytically active. We also wanted to compare the levels of enzyme activity and overall protein production to current industrial standard *E. coli* strains like BL21. We chose two industrially relevant enzymes which are currently produced using *E. coli* systems - β-galactosidase (encoded by the *lacZ* gene) and α-amylase (encoded by the *amyA* gene) (Walsh, 2018). β-galactosidase degrades a substrate called Chlorophenol red-β-D-galactopyranoside (CPRG) to produce Chlorophenol red which is a red dye (Paradis-Bleau et al., 2014). α-amylase degrades starch and hence starch-based assays can be used to test for enzyme activity.

We had access to the ASKA collection (Kitagawa et al., 2005; *NBRP Ecoli Strain - ASKA Collection*, n.d.) which consists of all *E. coli* genes based on the sequence of the K12 W3110 strain of *E. coli.* The host strain of the ASKA collection is *E. coli AG1* and has an auxotrophy for thiamine and histidine. The genes are stored in the pCA24N plasmid with a chloramphenicol resistance marker (Cm^R). The cloned ORF is under the control of the IPTG-inducible T5-lac promoter. Both *lacZ* and *amyA* genes were available in the ASKA collection.

A starch-agar plate assay was used to study the halo sizes formed by the α-amylase produced in different *E. coli* strains. Additionally, a 3,5-dinitrosalicylic acid (DNS) assay was also designed to test for α-amylase activity. DNS assays have been used to check

for the presence of reducing sugars and since α-amylase catalyzes the breakdown of starch into maltose, which is a reducing sugar, this assay can be used to estimate the activity of the enzyme based on the concentration of maltose formed. Alongside this, a CPRG-agar plate assay was developed to study the halo sizes formed by recombinant β-galactosidase produced by different *E. coli* strains.

This third part of the project also aims at understanding the functionality of the secretion systems in *E. coli* L-forms. We decided to study the effect of the addition of a secretion tag targeting the Sec Pathway, *pelB,* on our enzymes. The *pelB* gene encodes for periplasmic pectin lyase and is one of the secretion sequences used to secrete proteins in an unfolded state. This is also readily available in an inducible pET26b(-) plasmid system. To compare the effects of the presence and absence of this secretion tag on different strains, used *Escherichia coli* DH5a, *Escherichia coli* K12 MG1655, and *Escherichia coli* BL21 DE3 A1 as controls.

Chapter 2: Materials and Methods

Detailed information about materials and protocols can be found under Supplementary Materials.

Bacterial Strains and Growth Conditions

LPB is an osmoprotecitve liquid media, prepared with equal parts YEME and TSBS, used to grow L-forms. *E. coli L-form* cultures were grown in LBP supplemented with 25mM Magnesium Chloride and PenG400 at 30°C and 100rpm. *Escherichia coli* DH5a, *Escherichia coli* K12 MG1655, and *Escherichia coli* BL21 DE3 A1 were grown in LB at 37°C at 200rpm. Supplements, 1mM IPTG, and 1% Arabinose were added when required. When necessary, antibiotics were added to the media at the following concentrations: 25ug/ml chloramphenicol, 400ug/ml PenG, 100ug/ml ampicillin, 50ug/ml kanamycin, 50ug/ul apramycin.

Construction of mCherry and tdTomato with Cm^R

The pSB1C3 plasmids containing the constitutive *gapA* promoter, *tdTomato,* and *mCherry* genes were amplified and isolated from *Escherichia coli* DH5a. Each fluorescent gene was paired with the *gapA* promoter and cloned into a pSB1A3 plasmid using 3A assembly (*Assembly:3A Assembly - Parts.Igem.Org*, n.d.). *gapA* was restricted with EcoRI and SpeI, both fluorescent genes were restricted with XbaI and PstI and the backbone pSB1A3 plasmid was restricted with EcoRI and PstI. Ligation was performed using T4 DNA Ligase. The Cm^R gene from the pSB1C3 plasmid was used to replace the Amr^R gene in the pSB1A3 plasmids to create pSB1-Cm^R-*gapA-mCherry* and pSB1-Cm^R-*gapA-tdTom* plasmids. These were introduced into *E. coli* DH5a using standard methods for DNA manipulation and transformation. All materials and protocols can be found under supplementary material.

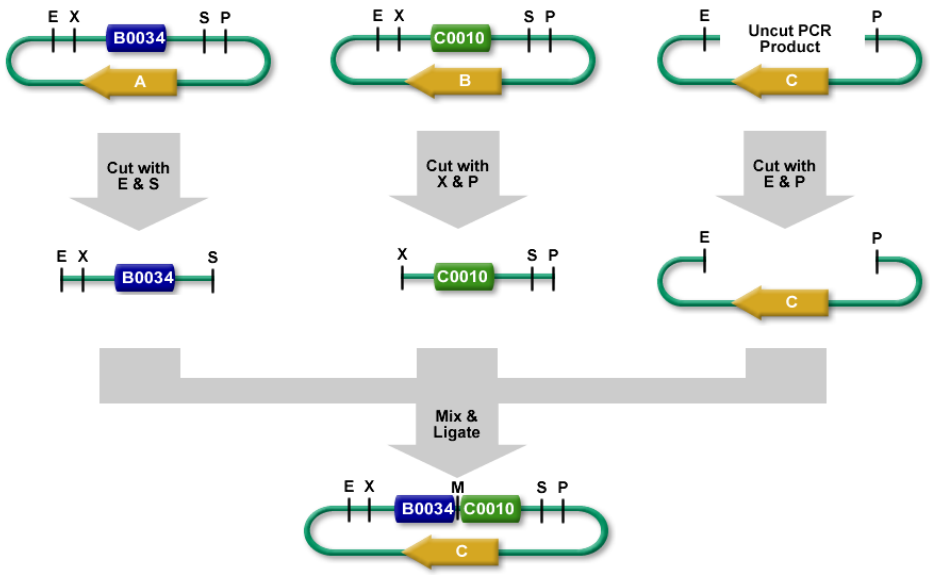


Figure 5: 3A Assembly. Reproduced from (*Assembly:3A Assembly - Parts.Igem.Org*, n.d.) Each fluorescent gene was paired with the *gapA* promoter and cloned into a pSB1A3 plasmid using 3A assembly. *gapA* was restricted with EcoRI and SpeI, both fluorescent genes were restricted with XbaI and PstI and the backbone pSB1A3 plasmid was restricted with EcoRI and PstI. Ligation was performed using T4 DNA Ligase.

Construction of IPTG inducible *amyA* and *lacZ*

Cm^R gene from the pSB1C3 plasmid was amplified by PCR with primers *CamR_fwd* and *CamR_rev* (Supplementary Table: Oligonucleotides). pET26b(-) Kan^R plasmid was used as the backbone to replace Kan^R with Cm^R using Gibson Cloning. Two fragments of the pET26b(-) vector were amplified (excluding the Kan^R coding sequence) by PCR using primers *pET26b_1_fwd, pET26b_1_rev, pET26b_2_fwd,* and *pET26b_2_rev.* Primers were designed using NEBuilder (*NEBuilder*, n.d.). Gibson reactions were performed with the three fragments to create the plasmid pET26b(-) Cm^R.

amyA and *lacZ* genes were amplified from pCA24N plasmids isolated from *Escherichia coli* AG1 (Kitagawa et al., 2005) by PCR using the primers *amyA-F-NcoI, amyA-F-NdeI, amyA-R-XhoI, lacZ-F-NcoI, lacZ-F-NdeI* and *lacZ-R-XhoI* respectively (Supplementary Table: Oligonucleotides). The primers were designed to include NcoI, NdeI, and XhoI restriction sites to enable the insertion of the genes into the pET26b(-) Cm^R plasmid backbone.

An error in reverse primer design for *amyA-R-XhoI* and *lacZ-R-XhoI* resulted in an addition of two extra base pairs before the 6xHisTag such that the HisTag was no longer in frame with the inserted gene. The next inframe stop codon added 96 excess base pairs (32 amino acids) to the end of each of the *amyA* and *lacZ* constructs. Out of the

four constructs, *pelB-lacZ* (with the secretion tag) could not be generated using the same restriction digestion cloning due to the presence of a double *NdeI* site in the *lacZ* gene sequence. This issue was noticed far too late into the project to correct for the design. Add double restriction site in resistance gene.

Successful insertion of the remainder of the constructs resulted in pET26b(-) *amyA* Cm^R, pET26b(-) *pelB amyA* Cm^R and pET26b(-) *pelB lacZ* Cm^R plasmids. These were introduced into *E. coli* DH5a, *E. coli* K12 MG1655 and *E. coli* BL21 DE3 A1 using standard methods for DNA manipulation and transformation.

Microscopic Imaging

Phase contrast microscopic imaging of samples was performed with the Zeiss PrimoStar Microscope. Fluorescence microscopy of samples was performed with the Zeiss LSM AiryScan Confocal Microscope.

DNA Isolation

Plasmid preparation was performed by first amplifying constructs in *E. coli* DH5a and then isolating them using either the Boiling preparation method (Supplementary Material) or the Nucleospin Plasmid EasyPure kit. 10-12 ml cultures of *E. coli* DH5a were pelletted for low copy plasmids like pET26b(-) and its derivatives while 5-6 ml cultures were used for high copy plasmids like pSB1 derivatives. Concentration of the plasmids was measured using a Nanodrop Spectrophotometer and plasmid constructs were verified on an agaore gel after restriction digestion using a standard restriction enzyme like HindIII. A Generuler™ DNA ladder mix was used as reference. PCR products were verified for their size and purified using a PCR DNA purification kit (Supplementary Table 2).

Genomic DNA was isolated from L-form *E. coli* cells using the Phenol-Chloroform Isoamyl alcohol (PCI) method. Concentration of isolated DNA was determined using a nanodrop spectrophotometer.

Bacterial Transformation

Walled and L-form strains of *E. coli* were transformed using a heatshock method. Competent cell stocks of walled strains of *E. coli* were prepared using a TSS buffer (Supplementary Materials - Protocols) and stored at -80°C until needed. Cells were exposed to a 90 second heatshock and ice treatment before and after the heatshock. Walled strains were recovered at 37°C after the addition of LB, and plated next to an open flame. Overnight (18 hours) incubation was performed at 37°C and colonies were counted on the next day.

Literature Review

A literature review was done with the intention of finding publications related to the industrial production of recombinant proteins in L-form and cell wall-deficient bacteria. This was crucial in understanding how L-forms can be created and proliferated, what kinds of growth conditions they require, and what they are currently known to be capable of. The findings have been listed under the results section.

Quantification and Statistical analysis

For Starch-agar plate and CPRG-agar plate experiments triplicates of each halo were used to calculate the average halo area, standard deviation, and standard error. Error bars represent standard error. Mean, standard deviation and standard error were used to estimate the magnitude, distribution, and error of an effect based on the data. Other measures of statistical significance were not used due to a limited number of experimental trials.

Definition of measures used:

Mean is the number obtained by adding several quantities together and dividing the sum by the number of quantities. Standard Deviation is the root mean square deviation about the mean. Standard Error is the standard deviation divided by the root of the number of samples.

Chapter 3: Results

L-form *E. coli* transforms efficiently under heat-shock conditions

E. coli L-forms cells have been known to be notoriously hard to transform with plasmid DNA. There was no established protocol for transforming wall-deficient cells of *E. coli* (Attempt #1, Table: Iterative changes to L-form *E. coli* Transformation Protocols) and relied on trial and error. The fragility of the wall-deficient cells prevented them from being made into competent cell stocks. In addition, L-form cells are naturally competent and are able to take up DNA with various methods (Kapteijn et al., 2022).
Hence, live cultures are used to prepare the cells for transformation.

The first attempts with transforming *E. coli L-forms* cells were with a high copy plasmid pSB1C3. For each transformation with an overnight preculture type, L-form *E. coli* cells were subcultured overnight and allowed to grow to their maximum OD of 0.6. These cells were aliquoted and spun down and tested with the transformation protocol. The reasoning was that most cells in an overnight culture are fully grown and alive, unlike a 2-3 day old culture, where the cells have already entered their death phase - making the overnight cultured cells more likely to take up external DNA from their environment.

Attempt #1 (Table: Iterative Changes to L-form *E. coli* Transformation Protocols) was plated with a cell spreader, as is common practice for walled strains of *E. coli.* However, due to their fragility, L-form cells most likely are not able to survive high amounts of shear stress. Attempt #2 attempted the transformation with higher concentrations of plasmid DNA without using a cell spreader, but instead, using a cotton swab for spreading the cells on the plate. Another trial was without using anything to spread the cells (Attempt #3), simply decanting the transformation mixture onto the plate and tilting the plate to spread out the mixture. All three of these attempts did not yield any transformed colonies. Hence to speed up the rate at which we tested different conditions, three more attempts were set up simultaneously (Attempt #4-6), each with a slight change in the protocol. Attempt #4 yielded two colonies of L-form *E. coli* with mCherry. Transformation with a tdTomato gene in a high copy pSB1C3 plasmid worked on Attempt #7 but the protocol was replicable.

The second set of transformations, i.e. Attempts #9-15 were done approximately 3 months after Attempts #1-8. At this point, it was necessary to understand why certain steps are performed in a standard heat shock transformation with chemically competent walled *E. coli* cells. Chemically competent *E. coli* are made in a TSS buffer (Materials and Methods) which consists of magnesium or calcium chloride, DMSO, and PEG. The presence of divalent cations in the mixture helps in counteracting the negative charge on the cellular membrane and adsorbing DNA molecules to it (Asif et al., 2017). Cycles of low temperature and high temperature (42°C) contribute to the loss of proteins and lipids from the cellular membrane, thus enlarging the pore size on the cell surface (Asif et al., 2017). Hence it is essential that cells are kept cool before the heat-shock. The low temperatures also contribute to restricting the fluidity of the lipid membrane, and this helps strengthen the cation’s interactions with the cell membrane.

Based on these findings, the protocol for L-form transformations was changed to include steps to cool down the cells on ice, introduce magnesium cations, and a heat shock. Attempts #11-15 indicate the trials with varying concentrations of magnesium chloride, varying times of ice incubation, and varying quantities of plasmid DNA. The transformation efficiency immediately increased with each attempt and the final protocol (Attempt #15) was replicable even with low copy plasmid transformations with pET26b(-). Based on the growth curve for L-form *E. coli* (unpublished data), the exponential growth phase starts around OD_{600} 0.2-0.3, with a maximum of 0.6 for a fully grown culture.

Table 1: Iterative Changes to L-form *E. coli* Transformation Protocols. (Each yellow highlight indicates a change in the protocol compared to the previous iteration. Each green highlight indicates transformations that yielded at least one colony. Transformation efficiency = Fraction of plates that yielded at least one colony. *OD Measurements at 600nm)

Protocol Steps	**Attempt #**							
	1	2	3	4	5	6	7	8
Preculture Type	Overnight	Overnight	Overnight	Overnight	Overnight	Overnight	Overnight	Overnight
Volume of culture utilised	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml
Centrifugate	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g
Reduce volume by removing supernatant to	50ul	50ul	50ul	50ul	50ul	50ul	50ul	50ul
Add plasmid DNA	mCherry in pSB1C3	mCherry in pSB1C3	mCherry in pSB1C3	mCherry in pSB1C3	tdTomato in pSB1C3	tdTomato in pSB1C3	tdTomato in pSB1C3	tdTomato in pSB1C3
Quantity of DNA	750ng	1000ng	1000ng	1000ng	1000ng	1500ng	1500ng	1500ng
Add PEG6000	200ul 5% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB
Incubate at 30°C	1 h	1 h	1 h	1 h	1 h	1 h	1 h	1 h
Heatshock at 42°C	-	-	-	60 s	-	-	-	-
Add LPB	250ul	250ul	250ul	250ul	250ul	250ul	250ul	250ul
Recovery at 30°C	1 hour	1 hour	1 hour	1 hour	1 hour	1 hour	1 hour	1 hour
Plating	Cell spreader	Cotton Swab	Decant	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant
Transformation Efficiency	0	0	0	1/8	0	0	4/4	0
Number of colonies	0	0	0	2	0	0	7	0

Protocol Steps	Attempt #						
	9	10	11	12	13	14	15
Preculture Type	Overnight	Overnight	Overnight	Overnight	OD* 0.2-0.3	Overnight	OD* 0.2-0.3
Volume of culture utilised	1ml	1ml	1ml	1ml	1ml	1ml	1ml
Centrifugate	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g	3min @ 2700g
Reduce volume by removing supernatant to	50ul	50ul	300ul	300ul	200ul	300ul	300ul
Keep on Ice	-	-	-	2min	-	2min	2min
Add plasmid DNA	pET + AmyA or LacZ	pET + AmyA or LacZ	pET + AmyA or LacZ	pET + AmyA or LacZ	pET + AmyA or LacZ	pET + AmyA or LacZ	pET + AmyA or LacZ
Quantity of DNA	1000 ng	1500 ng	20,000 ng	20,000 ng	20,000 ng	1,000 ng	1,000 ng
Add PEG6000	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB	200ul 25% in LPB
Add MgCl2 (2.5M)	-	-	330 ul	100 ul	16.6ul	68 ul	68 ul
Final molarity of mixture	-	-	1M	0.4M	0.1M	0.3M	0.3M
Incubate on Ice	-	-	-	-	-	30min	30min
Incubate at 30°C	1 h	30 min	1 h	1 h	1 h	-	-
Heatshock at 42°C	-	-	30 s	30 s	-	30 s	30 s
Keep on Ice	-	-	-	2min	-	2min	2min
Add LPB	250ul	250ul	250ul	250ul	250ul	400ul	400ul
Recovery at 30°C	1 hour	1 hour	1 hour	1 hour	1 hour	1 hour	1 hour
Plating	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant	Cotton Swab/Decant
Transformation Efficiency	0.00	0.00	0.00	3/6	0.00	5/6	8/8
Number of colonies	0	0	0	9	0	19	56

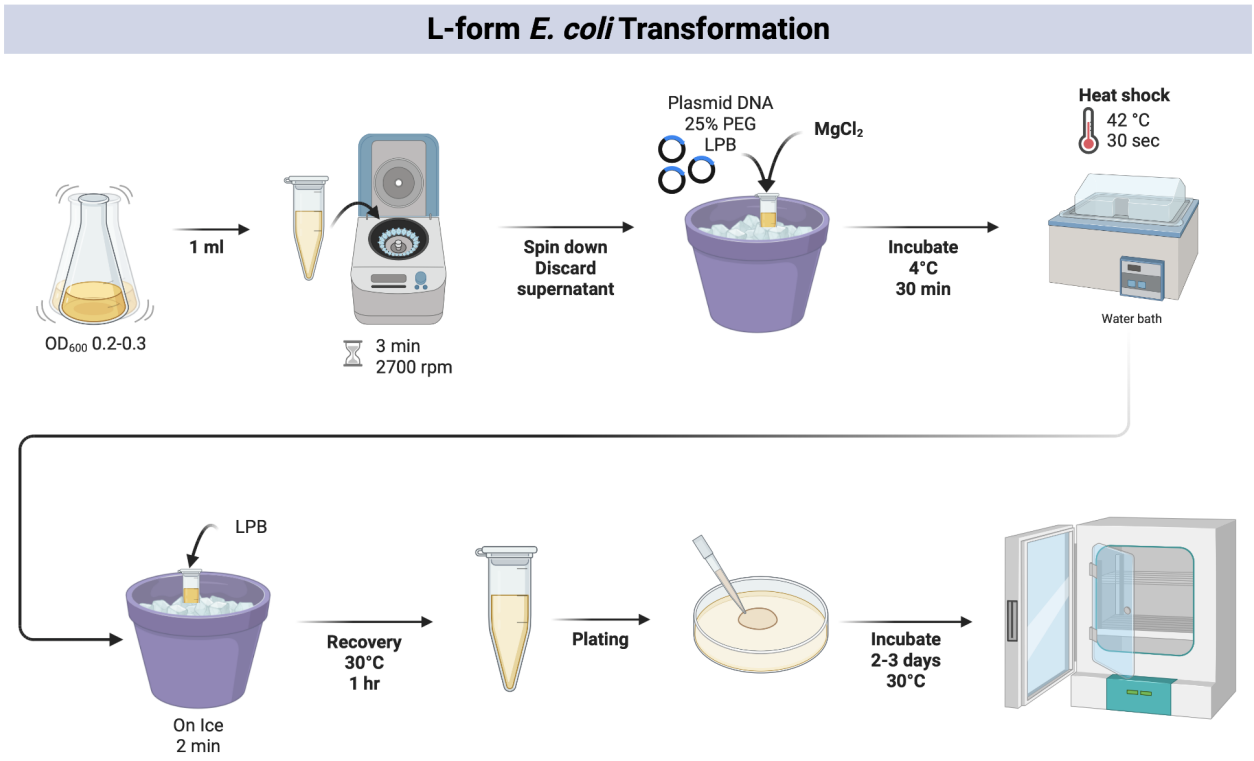


Figure 6: L-form *E. coli* transformation protocol using a heatshock and ice incubation. This protocol depicts Attempt #15 from Table 1. Made with Biorender

L-form *E. coli* can revert back to walled forms

The phenomenon of L-form switching (Kawai et al., 2018; Mickiewicz et al., 2019; Petrovic Fabijan et al., 2022) has been shown to have clinical and therapeutic significance. It is the ability of L-form cells to adapt to their environment by "switching" to a walled form of cells. In their walled forms, these cells are easier to store long-term as glycerol stocks and are not as fragile as their wall-deficient counterparts.

To aid in creating long-term stocks of the mCherry and tdTomato strains of *E. coli L-forms* we prompted them to revert back to their walled forms by first subculturing them twice into LB media and growing them at 37°C 100 rpm. Cells were then streaked onto LB plates and grown overnight at 37°C. Restreaking a second time onto LB resulted in the WT strain and tdTomato strain showing walled forms of the cells, which are visually different on LB plates than the wall-less forms (Figure 7).

Both L-form strains with mCherry, however, did not show any emergence of walled forms. These cells were further restreaked on LB plates for three more iterations, without success.

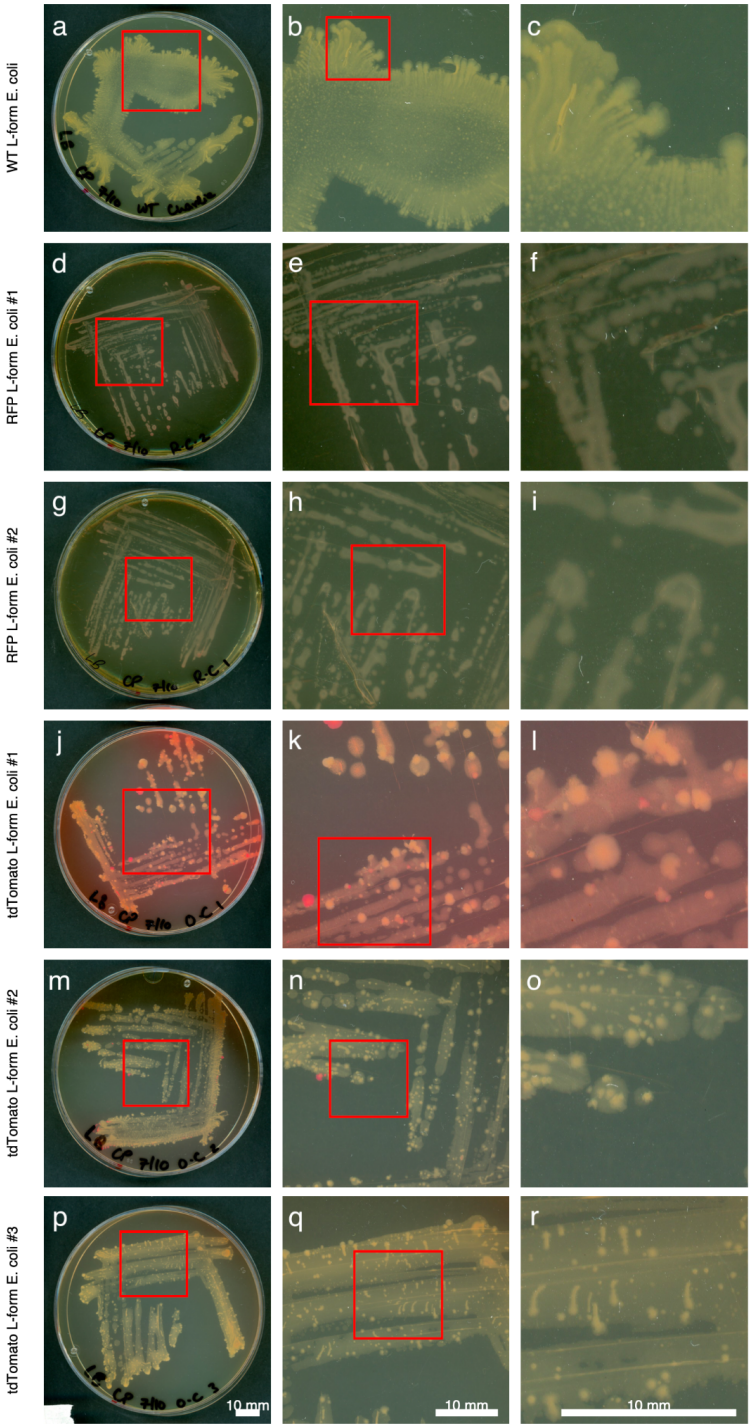


Figure 7a: Differences in growth of L-form *E. coli* cells as Wild Type, or with pSB1C3 plasmid containing mCherry or tdTomato genes. (a-c) Wild Type *E. coli* L-forms. White outgrowth resembles walled cells with rod morphology growing. (d-i) Two replicate plates of L-form *E. coli* transformed with pSB1C3 plasmid containing Cherry gene. Unlike the a-c and j-r, these do not have walled, rod-morphology shapes arising from their plates. (j-r) Three replicate plates of L-form *E. coli* transformed with pSB1C3

plasmid containing tdTomato gene. Clear white outgrowths can be observed like the wildtype a-c indicating walled, rod morphology cells growing.

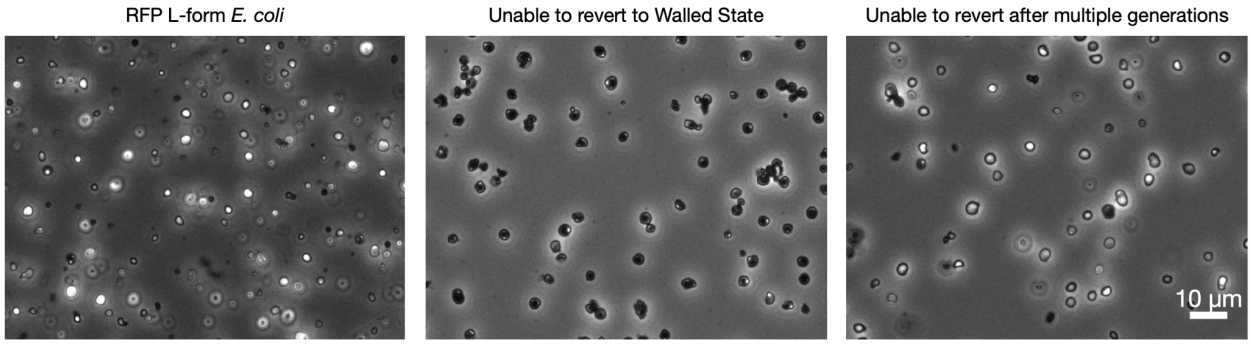


Figure 7b: No L-form switching observed. L-form *E. coli* transformed with pSB1C3 plasmid containing mCherry gene unable to revert back into the walled forms after five iterations of LB plate subcultures.

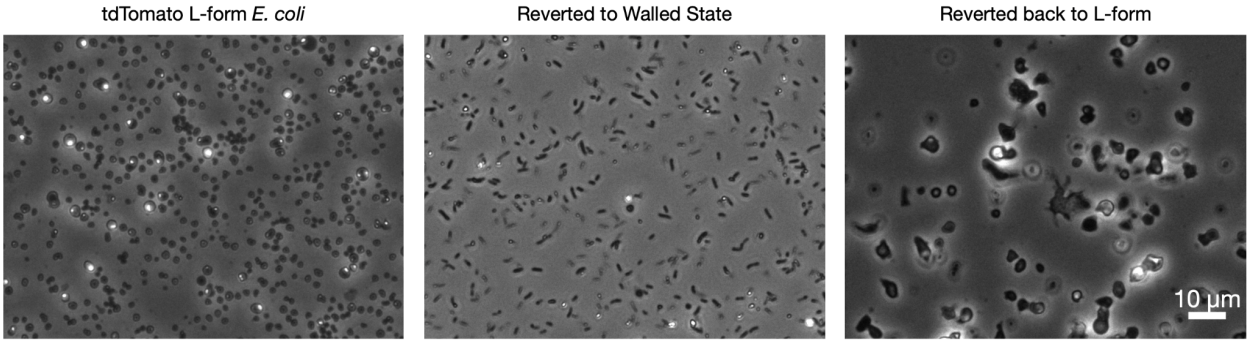


Figure 7c: L-form switching. L-form *E. coli* transformed with pSB1C3 plasmid containing tdTomato gene. Cells are able to revert back into a walled form and also switch back into an L-form after growth in LPB with PenicillinG.

Protein production rates of L-form *E. coli* differ based on growth conditions

E. coli L-forms cells usually take 2-3 days to start growing properly on LPMA agar plates at 30°C and start showing color if they contain a fluorescent gene like mCherry or tdTomato. However, when plated on LB at 37°C for L-form switch experiments, they were able to show color, i.e. express fluorescent proteins within 18 hours. This was a unique observation and prompted us to design an experiment to test out the various conditions under which *L-form E. coli* cells could optimally produce proteins. These experiments were, however, only limited to plate-based growth conditions.

The main variables to be tested in this experiment were the Temperature of incubation, Media on which cells are grown - LB vs LPMA (supplemented with magnesium chloride and horse serum), and the presence or absence of PenicillinG. One wild-type L-form, two mCherry L-forms, and three tdTomato L-form strains were tested out on the above

conditions, grown overnight for 18 hours, and imaged. The intensity of color shown by each sample would be used to determine the amount of protein produced by the strain. Cells containing mCherry appear red and those containing tdTomato appear orange-pink.

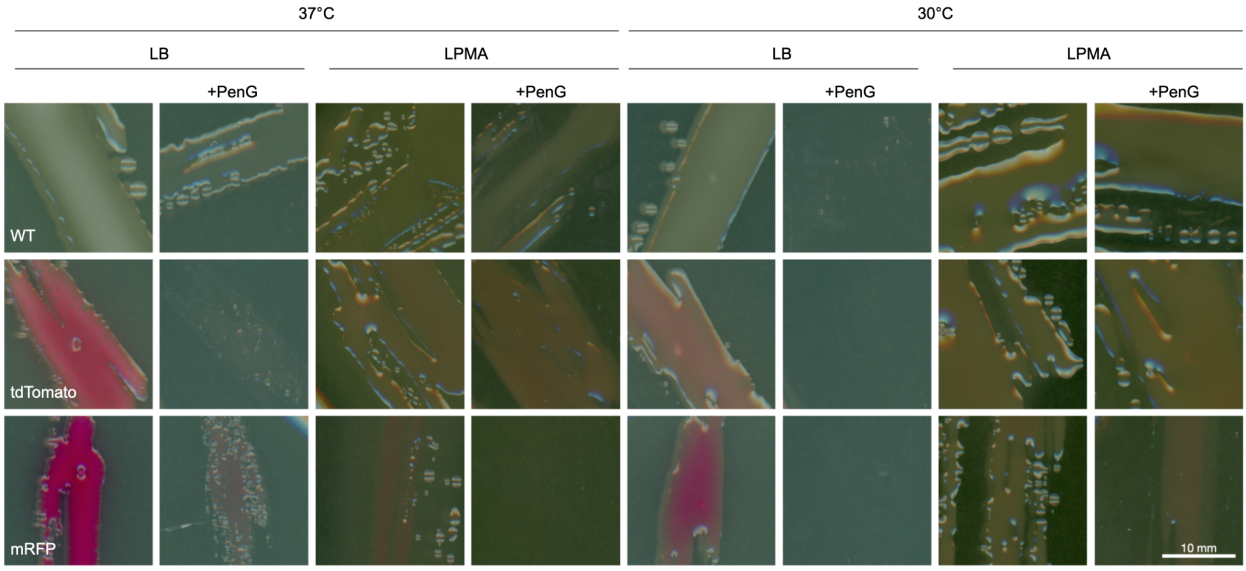


Figure 8: *E. coli L-forms* cells and conditions for protein production. WT, mCherry, and tdTomato expressing cells grown under different Temperatures, Media, and PenG conditions.

LB media without PenG, incubated at 37°C makes the cells produce the highest amount of protein, as indicated by the intensity of color seen. LB media without PenG incubated at 30°C also shows intense color but much less than 37°C plates.

Cells have been known to produce excess amounts of proteins when stressed and L-form cells of *E. coli* are stressed when grown in a non-osmoprotective media like LB. The addition of PenG drastically reduces the quantity of proteins produced as indicated in Figure 8. Experiments like cell proliferation and apoptosis assays can be performed to check and quantify the stress that the cells are experiencing.

L-form *E. coli* are capable of expressing fluorescent proteins

As a part of the first module of experiments, we wanted to test whether L-form *E. coli* cells were capable of producing properly folded simple proteins. Fluorescent proteins are perfect for this purpose. *E. coli L-forms* cells transformed with pSB1C3 plasmids containing mCherry and tdTomato fluorescent protein coding sequences were imaged with the Zeiss LSM AiryScan Confocal Microscope under split channels.

It is clear that the cells are able to express functional fluorescent proteins. They are also producing *gfp* proteins constitutively along with the plasmid-inserted fluorescent proteins (Figure 9). Three cell lines of L-forms with tdTomato and two cell lines of L-forms with

mCherry were created and imaged. We were able to show that L-form *E. coli* are capable of producing fluorescent proteins. We used mCherry and tdTomato which are both derived from Discosoma sp. (Shaner et al., 2004). tdTomato is much larger in size and mass (54.2 kDa) as compared to mCherry (26.7 kDa). Both proteins need to be folded properly in order to be functional and express fluorescence. This shows that the partial or complete loss of the cell wall in L-form cells doesn't necessarily affect the process of protein folding.

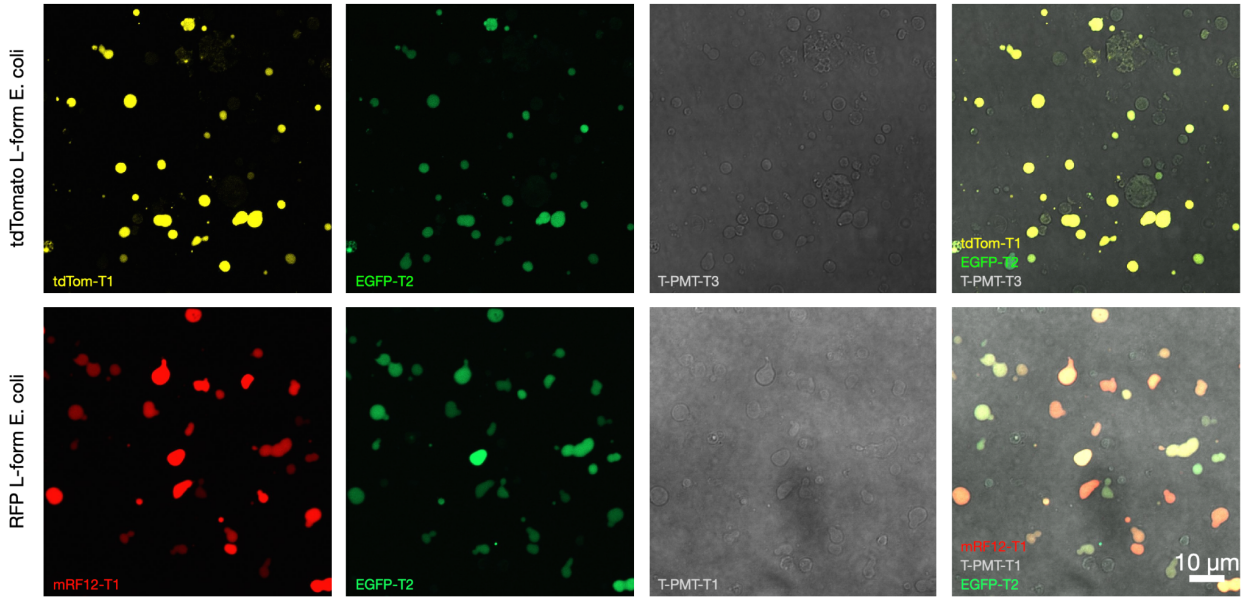


Figure 9: Fluorescent proteins expressed in L-forms of *E. coli*. L-forms of *E. coli* K12 MG1655 transformed with tdTomato and mCherry genes with a strong constitutive GapA promoter. The strain has an eGFP gene incorporated into its genome.

Viability of Starch and CPRG Agar plate assays was confirmed with walled *E. coli*

Before testing whether L-form *E. coli* are able to produce functional enzymes or not, the viability of the agar plate assays for the two enzymes was tested using the BL21, DH5a, and MG1655 strains of *E. coli.* The plate-based assays were successful, and the thresholds for halo detection from each strain were met after spotting live cells and sonicated protein samples.

These assays were optimized for the number of samples that would be tested at a time, and plates of appropriate size were chosen. Culture volumes that were used to prepare the sonicated samples were also optimized to yield halo sizes in a range that was detectable. Having no variation in halo sizes would not yield any results, and hence finding the right amount of culture to use was essential. There is an upper and lower limit to the amount of enzyme that a sample can have in order to have variability in halo sizes. Having too little enzyme would result in no halo formation, and having too much

enzyme would show no variation in halos from different cultures. The optimal culture volume and spotting volume were determined after four trials of both CPRG and Starch agar plate assays. 10ml cultures were pelleted down and sonicated in 700ul of PBS. 5ul of this mixture was spotted on each agar plate for the “Sonicated Sample” plates. At the same time, 5ul of the live, 4-hour-induced culture was spotted for the “Live Sample” plates. These were found to be optimal in detecting differences in halo sizes.

Positive control tests with native α-amylase and β-galactosidase were partially successful

A Starch-LB agar plate assay was performed with *Escherichia coli* BL21 DE3 A1 expressing *amyA* in the pCA24N plasmid as a positive control. Due to the error in the design of the reverse primers *amyA-R-XhoI* and *lacZ-R-XhoI* (Table 3: Oligonucleotides), the ORFs for *amyA* and *lacZ* cloned into the pET26b(-) plasmid have been extended by 96 bps, adding an additional 32 amino acids to the end of the protein.

To test whether the modified gene sequences had a similar enzyme activity to the native sequences from the ASKA collection, both *amyA* and *lacZ* were expressed using the pET26b(-) vectors (with the excess amino acids) and the pCA24N vector as the control (with the native enzymes). Protein overexpression was performed with IPTG and arabinose induction as stated under Methods. The results were quite surprising. The native α-amylase enzyme did not seem to function while the altered enzymes expressed in the pET26b(-) vectors were catalytically active in degrading starch (Figure 10a) as seen by the transparent halos on the starch-agar plate. The CPRG-agar plate yielded halos for both the native enzyme expressed using the pCA24N plasmid and the altered enzyme expressed using the pET26b(-) vector (Figure 10b). This indicates that the altered β-galactosidase enzyme is still catalytically similar to the native version and is capable of degrading CPRG.

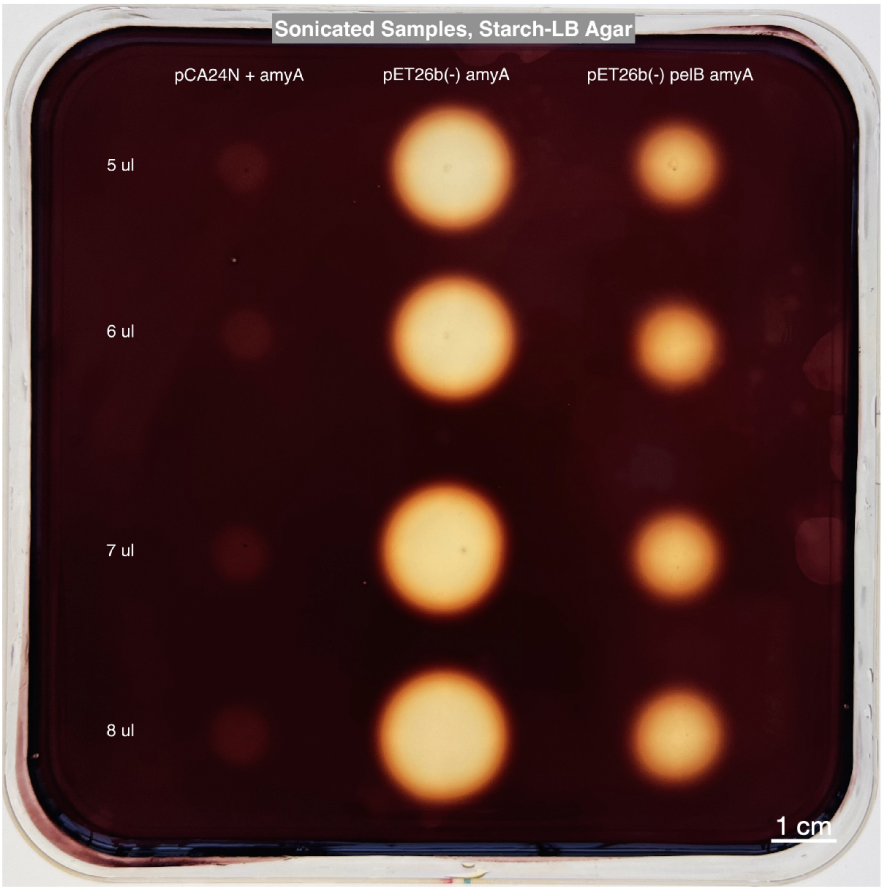


Figure 10a: *amyA* positive control shows no activity. Starch-Agar plate assay stained with Gram's Iodine. *amyA* was overexpressed in *Escherichia coli* BL21 (details in Supplementary Table 1) in the native ASKA plasmid pCA24N, in pET26b(-) and pET26b(-) *pelB*, sonicated and spotted in each column from left to right respectively. Increasing volumes of sonicated samples were spotted in each row from top to bottom respectively. Native pCA24N *amyA* shows no activity compared to *amyA* expressed in the pET26b(-) vectors.

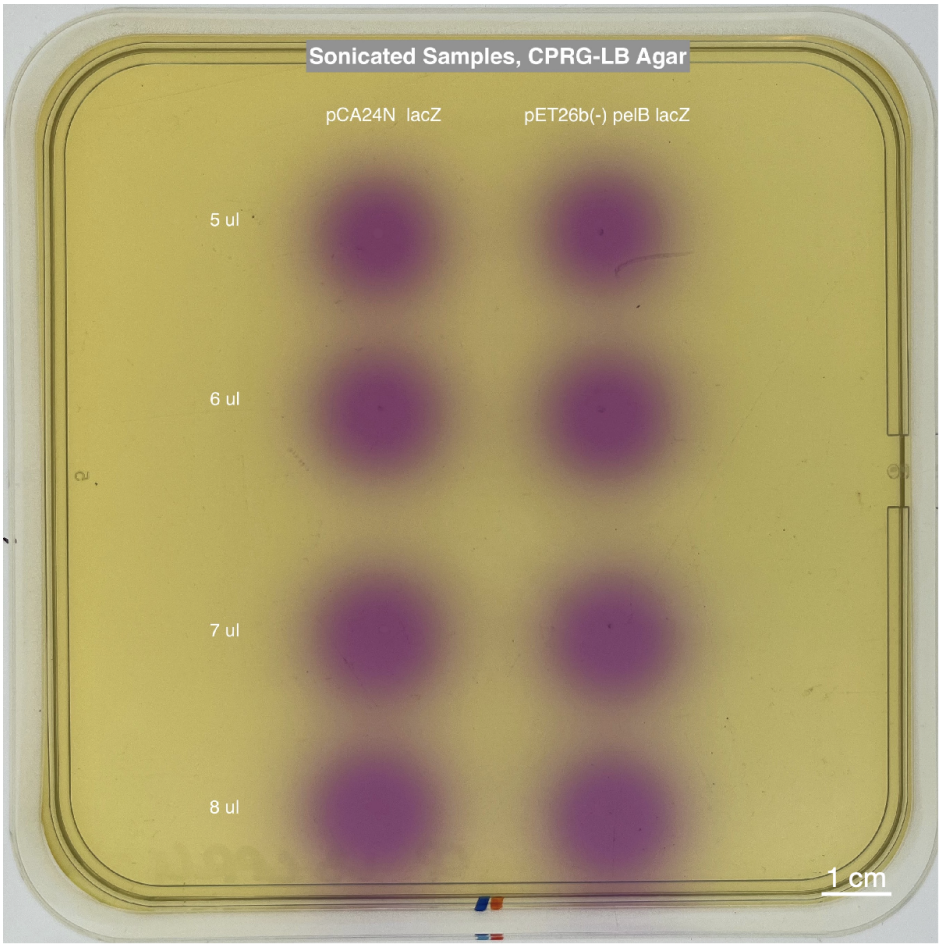


Figure 10b: Modified enzyme has similar activity to positive control. CPRG-Agar plate assay. *lacZ* was overexpressed in *Escherichia coli* BL21 DE3 A1 in the native ASKA plasmid pCA24N and pET26b(-) *pelB*, sonicated and spotted in each column from left to right respectively. Increasing volumes of sonicated samples were spotted in each row from top to bottom respectively. *lacZ* expressed in *pET26b(-) pelB* shows similar activity compared to *lacZ* expressed in the native pCA24N vector.

L-form *E. coli* can produce functional enzymes

As a part of the second module of experiments to test whether *E. coli L-forms* cells are capable of producing complex and functional proteins like enzymes, β-galactosidase and α-amylase were expressed using the *lacZ* and *amyA* genes obtained from the ASKA collection (Kitagawa et al., 2005; *NBRP Ecoli Strain - ASKA Collection*, n.d.).

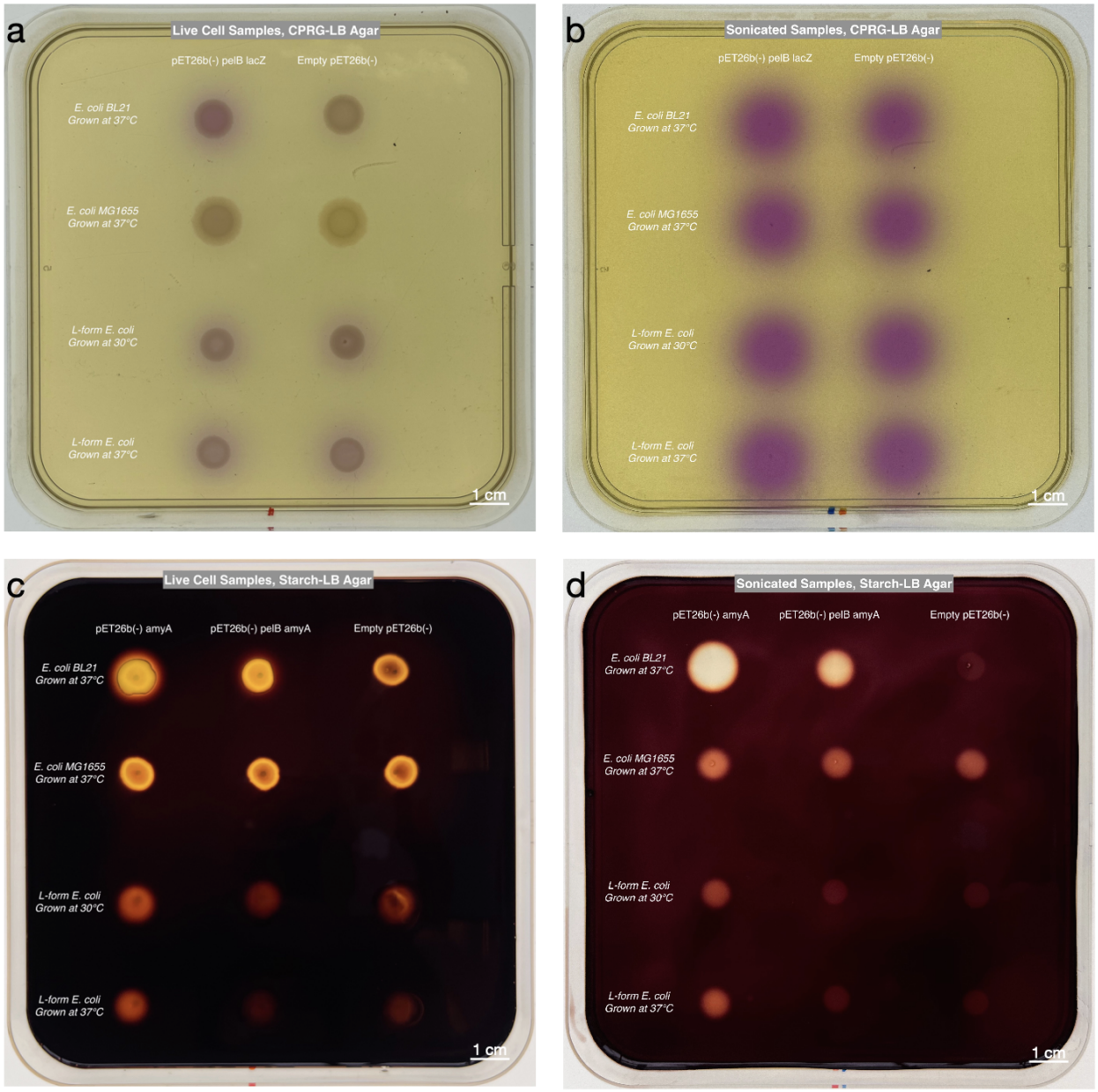


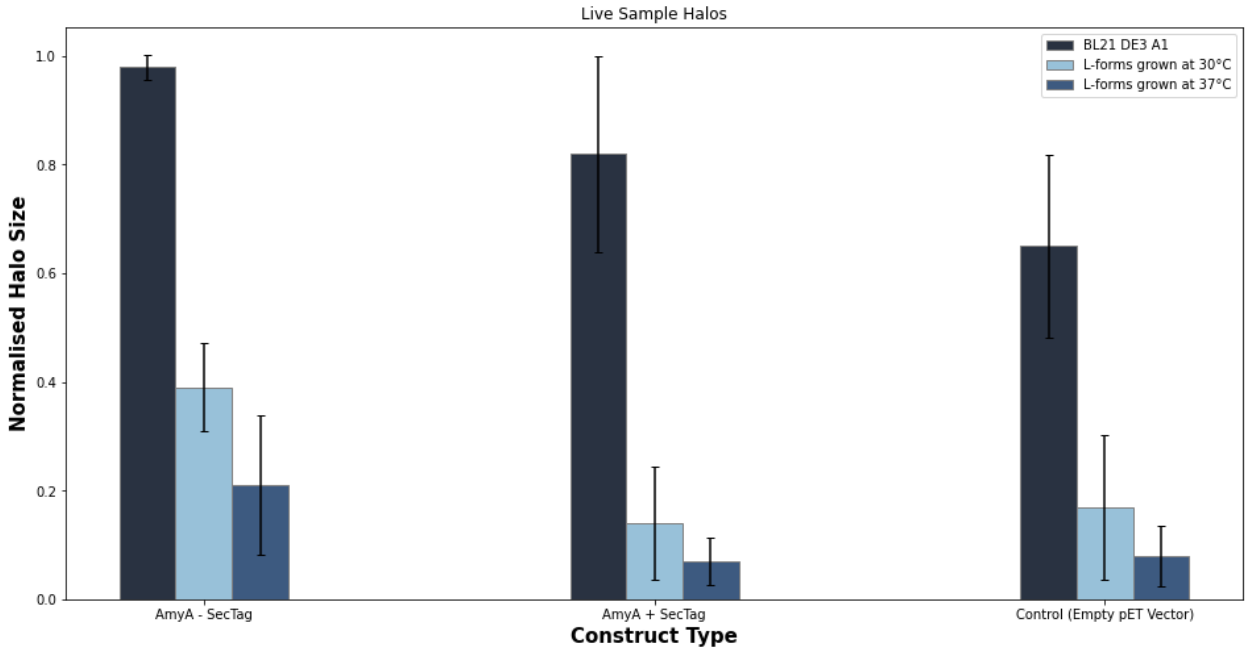
Figure 11: Functional enzymes produced and visualised on Agar plate assays. (a,c) *Escherichia coli* strains BL21, MG1655 and L-forms have been grown on LB overnight and imaged after 18 hours of incubation. (b,d) Sonicated samples from the same strains have been spotted onto CPRG and Starch agar plates respectively and incubated at 37°C for 18 hours. L-form *E. coli* grown at 30°C refers to the protein overexpression temperature of 37°C.

E. coli L-forms clearly show enzyme activity for both β-galactosidase and α-amylase as indicated by the red and transparent halos on the bottom two rows of each CPRG and Starch agar plate (Figure 11).

It has been observed that CPRG agar plate assays yielded similar halo sizes for both the negative control (empty pET26b(-) vector) and the test strain (pET26b(-) *pelB lacZ*) in the sonicated samples (Figure 11b). Due to this, quantitative analysis of the enzyme activity cannot be performed.

The presence of a *pelB* secretion tag lowers α-amylase activity in Starch-Agar assay

It has been observed that the halo sizes for the *E. coli* strain BL21 and the L-form strains overexpressed at 30°C and 37°C are lowered in the presence of a *pelB* secretion tag. This result can be seen in both Live cell samples as well as sonicated samples (Figure 12). Comparatively, the halo sizes for *amyA pelB* in L-form strains are much smaller than the halo sizes in walled strains.



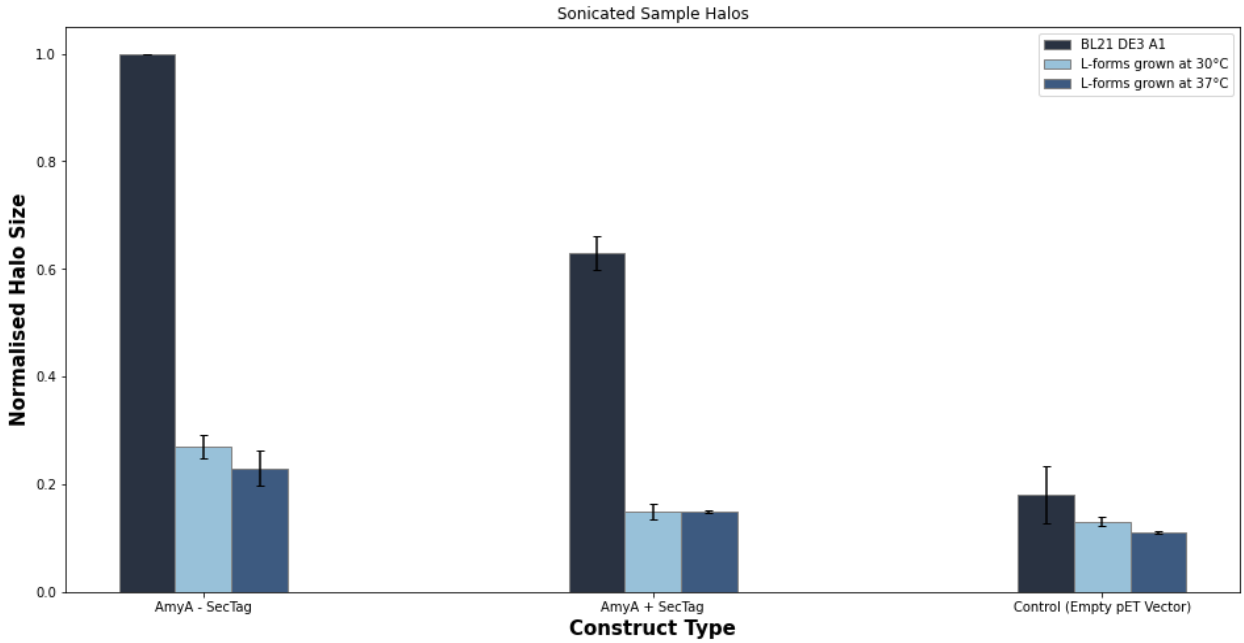


Figure 12: Halo size comparison for Starch Agar plates. Halo size of each amyA construct expressed in different strains of *E. coli* at different temperatures, as indicated by the legend. Normalisation has been done for each plate with the maximum halo size for that individual agar plate. All area measurements have been done with ImageJ (FIJI).

***Escherichia coli* K12 MG1655 showed no variation in halo sizes for test vs control**

The parent strain of L-form *E. coli,* i.e. MG1655, shows an equivalent expression of both β-galactosidase and α-amylase against the control of an empty plasmid. The levels of protein observed in the sonicated samples of MG1655 are varied, but the enzyme activity observed on Starch-LB and CPRG plates of AmyA and LacZ, respectively, compared to pET without either insert are relatively the same as indicated by the normalized halo areas.

Starch and CPRG agar assays with supernatant samples show no halo formation

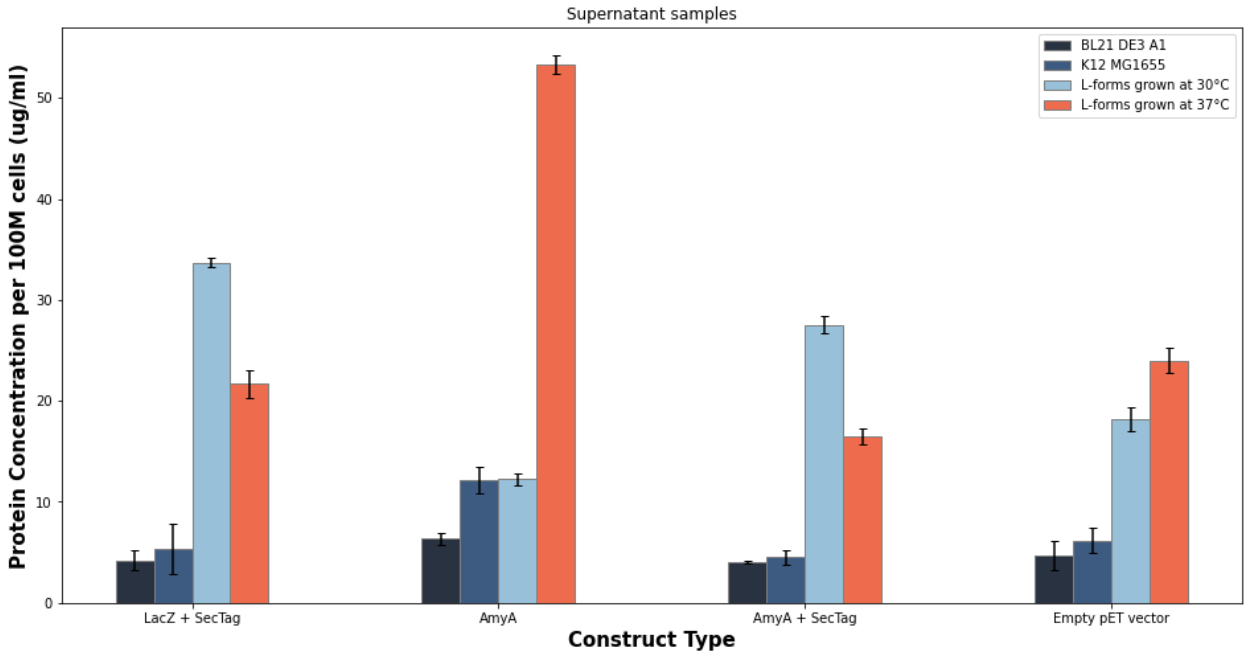
Starch and CPRG agar plates show no enzyme activity for both α-amylase and β-galactosidase. The protein concentration in the supernatant is below the detection threshold for halo-based plate assays. The protein concentration for the supernatant is in the range of 8.3-80.9 ug/ml as indicated by quantification studies using the Bradford assay.

L-form E. coli produce proteins within the same order of magnitude as the industrial standard strain

Protein quantification has been done with the help of a Bradford assay using the same supernatant and sonicated samples used to set up the Starch and CPRG agar plate assays. The BSA standard curve can be found under Supplementary Methods.

L-form *E. coli* cells are morphologically bigger than walled forms and thus are expected to produce more amount of protein for the same number of cells. This is apparent from the normalised values of protein production per 100 Million cells. Normalisation was performed using OD_{600} values of each strain at induction using IPTG. Unpublisehd OD vs Number of cells data for L-form *E. coli* was used to calculate the number of cells. Figure 13 indicates this trend as the quantity of protein produced in L-forms is more than both of the walled strains (BL21 and MG1655 *E. coli*). Overall protein concentrations of the *E. coli L-forms* strain are in the same order of magnitude as the concentrations of the *Escherichia coli* BL21 DE3 A1 strain, which is the industry standard. These results paired with the observations from the Starch agar plate assay indicate that the BL21 strain is able to produce more functionally active protein for similar overall protein concentrations as compared to the L-form strain.

amyA expressed without a *pelB* secretion tag has higher concentrations than *amyA* expressed with a *pelB* secretion tag in the supernatant and at the same time it has lower concentrations in sonicated samples than *amyA* expressed with a *pelB* secretion tag. This is contrary to what might be expected. This observation paired with the halo sizes observed in the Starch agar plate assay (*amyA* without *pelB* has a larger halo in almost all cases than *amyA* with *pelB*) implies that *E. coli* strains expressing α-amylase without any secretion tags produce a much higher concentration of active enzyme as a percentage of total protein produced within the cells.



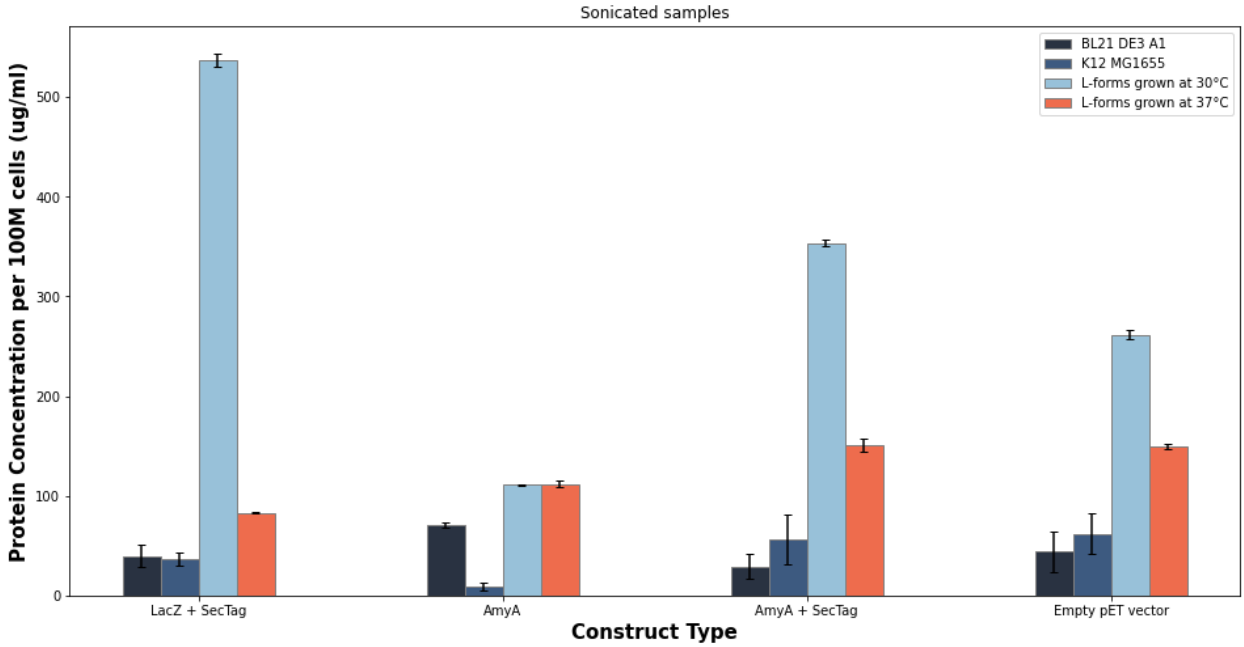


Figure 13: Total protein concentration measured in supernatant samples and 10x diluted sonicated samples.

Chapter 4: Discussion

General properties of L-form *E. coli*

Due to their fragility, L-form cells cannot be preserved as glycerol stocks at -80°C. They have to continually be subcultured every 4-5 days to ensure that the cultures remain alive. A precaution that needs to be taken is to be careful about mycococcus contaminations in the L-form strains of E. coli. Due to their coccus morphology, they appear similar to L-form E. coli cells. If there is such a doubt, 16S sequencing should be performed to verify the cell line.

Some strains of L-form *E. coli* aren't able to undergo an L-form Switch

The *E. coli mCherry* (L-form) strain was unable to undergo an L-form switch, unlike the WT and the tdTomato strains. One possibility is that both of the strains came from the only two transformed colonies that were visible after Transformation Attempt #4 (Table 10). There were only two colonies, and both cultures were unable to undergo a switch. It might have been likely that the cells had significant mutations that allowed them to take up the mCherry plasmid, but there is no way of knowing for sure what the cause of this is without performing further experiments.

Strains that are unable to revert back to the walled form need to continuously be subcultured after every few days to keep them proliferating.

Copy number of the pET26b(-) plasmid

The pET26b(-) plasmid has a very low copy number, which means that there are very few copies of the plasmid that exist within each cell. The copy number has implications on the rate of protein synthesis and also the growth rate of the cell under antibiotic selection. Choosing a plasmid with a higher copy number might have had an impact on the transformation efficiency of L-form *E. coli.* Since there would have been more copies of the plasmid within each cell, they would have had a higher chance of survival under antibiotic stress. But this needs further research.

Flaws in cloning design and α-amylase liquid assays

Two major flaws were noticed in the cloning design. The first flaw was overlooking the presence of a NdeI restriction site in the *lacZ* gene. The modified pET26b(-) plasmid only has one unique restriction site upstream of the *pelB* secretion tag: NdeI. So, the only option to create a construct without the secretion tag would be to digest with NdeI on one end. The *amyA* gene does not have a NdeI site in the gene, and hence it was possible to create a construct with amyA and without pelB. However, due to the presence of the NdeI site in the lacZ gene, the gene was restricted before it could insert into the plasmid. This was noticed towards the end of the thesis project and hence could not be corrected for using another method of cloning like Gibson Cloning or Restriction Free Cloning. This led to an incomplete set of pET26b(-) constructs, and hence β-galactosidase assays could not be used to study the effects of a secretion tag on enzyme activity.

The second flaw was concerning the pair of reverse primers (amyA-R-XhoI and lacZ-R-XhoI from Table 3) designed to amplify the respective genes from the pCA24N plasmids and add an XhoI restriction site to the ends of the genes. The primers provided by the ASKA website (*PEC W3110 (Profiling of E.Coli Chromosome) LacZ*, n.d.; *PEC W3110(Profiling of E.Coli Chromosome) AmyA*, n.d.) have three additional base pairs that do not match with the gene construct. Regardless of the circumstances, simulations of the cloning process using programs like Snapgene should have been performed before ordering primers as both of the above flaws could have been avoided.

With regards to the DNS assay to quantify the α-amylase concentration in different strains, a pilot run was performed where colorimetric analysis was performed in 1ml cuvettes (unpublished data), and a standard curve was obtained. In order to accommodate a much larger sample size, the assay was miniaturized to a 96-well plate. However, the standard curve readings from this assay were unreliable and the absorbance values of each standard sample in this miniaturized assay did not reflect the variation in the amount of maltose present in the sample. Due to the lack of a standard curve, the measured absorbance values of each sample could not be analyzed.

***Escherichia coli* K12 MG1655 showed no variation in halo sizes**

Despite having different constructs introduced into the strain, the overall size of the halos on the Starch agar plate assay and the CPRG agar plate assay remained approximately equal between different constructs. This could imply that there was not any significant effect of an additional gene coding for α-amylase or β-galactosidase on the strain.

The presence of a *pelB* secretion tag lowers α-amylase activity in Starch-Agar assay

Agar plate halo assays depend on diffusion as their main mechanism. A higher protein concentration implies that there is more spread. Also higher the activity of the enzyme, the more the substrate gets consumed. This could have an influence on the enzyme's catalytic activity. Improper folding of the protein could result in lowered enzymatic activity and, thus a smaller halo size on the starch agar plate. Another possibility is that the presence of the secretion tag makes the protein slightly bigger and thus takes more time to transcribe and translate. Since all strains have been grown for the same amount of time, longer manufacturing times could imply a lower yield of protein, also contributing to a smaller halo size.

Comparatively, the halo sizes for *amyA pelB* in L-form strains are much smaller than the halo sizes in walled strains. This could be because of dysfunctional secretion systems. Since secretion systems are dependent on a large number of membrane proteins, and since L-forms have a disrupted cell wall, which might affect the stability of the cell membrane, it could be that the Sec Pathway might not be as functional as in walled forms.

Positive control tests with native α-amylase and β-galactosidase were partially successful

There could be several reasons that the α-amylase produced using the pCA24N plasmid did not yield halos of the expected size. There could have been mutations induced in the plasmid while isolating it from the host ASKA strain (AG1) or the transformations into *E. coli BL21* might not have worked properly. Genes amplified from the ASKA plasmids (pCA24N) were verified on a gel to make sure that a DNA fragment of the right size was being used. However, plasmids were not isolated from BL21 after transformation to verify whether the strain had actually picked up the intended plasmid or not. So far, other labs (*Barrick Lab :: ReferenceKeioASKACollection*, n.d.) have utilized the ASKA collection, and no concerns regarding the amyA gene have been reported yet, implying that the enzyme should theoretically be functional.

CPR Halo sizes of test vs control samples are approximately equal

The size of the halos being approximately the same in the CPRG assay makes it very difficult to interpret the variations in each *E. coli* strain's ability to produce the functional enzyme. A closer look at the publication from (Paradis-Bleau et al., 2014) reveals that the observed CPR color development is directly proportional to the incubation time and that the color diffuses in the agar, easily spreading to areas where the enzyme has not reached. According to (Paradis-Bleau et al., 2014), early time points should be used for quantification. Another finding is that incubation of CPRG overnight at 37°C leads to excessive background color development, and thus the plates cannot be deemed useful.

There is a strong possibility that the β-galactosidase enzymes produced from different strains were yielding different-sized color halos, but due to the long incubation times (4-18 hours) at 37°C, the color was simply diffusing everywhere. It is important to mention that both of the enzymes that have been chosen are native to *E. coli* and that there will always be basal levels of expression regardless of an extra copy of the gene being introduced with a plasmid. Hence, incubation time should be drastically reduced before repeating this assay.

Another possibility would be designing a liquid based CPRG assay, where the amount of CPRG broken down is proportional to the amount of β-galactosidase enzyme added. Here the rate of the reaction is dependent on the amount of enzyme and hence can be easily quantified and standardized. This system would be been easier to analyze than agar plate halo-based analysis.

Conclusion and Future Steps

In conclusion, it appears that the strain *E. coli L-forms* is capable of producing proteins, simple and complex, and also is able to function under standard DNA manipulations and transformation conditions. A future step towards establishing L-form *E. coli* as an industrially viable strain would be to create a stable line of L-forms from *Escherichia coli* BL21 DE3 A1. This thesis aimed at comparing a K12 MG1655 L-form's abilities of protein production to that of an industrial standard strain which has been largely

optimized for being able to produce high amounts of quality proteins. Perhaps having those optimizations in an L-form would be highly beneficial. Another future avenue of research could be to explore methods of scaling up stable L-form cell lines to large-scale incubators without the use of antibiotics. Using antibiotics on a large scale poses major biosafety concerns and should be avoided.

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Supplementary Material

Key Resource Tables

Supplementary Table 1: Bacterial Strains

Reagent or Resource	**Source**	**Identifier**
Escherichia coli DH5a	Lab Stock	N/A
Escherichia coli K12 MG1655	Lab Stock	N/A
Escherichia coli K12 MG1655 *gfp*$^{+}$ *PenG*^{R} (L-form)	Lab Stock	E. coli L-forms
Escherichia coli BL21 DE3 A1	Lab Stock	N/A
Escherichia coli AG1 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_k^- m_k^+) *supE44 relA1*	(*NBRP Ecoli Strain - ASKA Collection*, n.d.)	ME5305

Supplementary Table 2: Chemicals, Peptides, Recombinant Proteins, Kits

Reagent or Resource	Source	Identifier
Chloroform	Honeywell - Riedel-de Haën	1731042
Glycogen	Thermo Fisher	N/A
Magnesium Chloride	Duchefa Biochemie	M0533.1000
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	67-68-5
LB Premix	Becton Dickinson	N/A
TSBS Premix	Becton Dickinson	N/A
YEME Premix	Becton Dickinson	N/A
Iberian Agar	Becton Dickinson	N/A

Poly(ethylene glycol) (PEG6000)	Sigma-Aldrich	P4338-500G
Horse Serum	Thermo Fisher	N/A
Lysozyme from chicken egg white	Sigma-Aldrich	12650-88-3
Isopropanol	VWR International	N/A
Ethanol	VWR International	N/A
Tris-HCl with EDTA	Thermo Fisher	N/A
Phenol (saturated with 10 mM Tris pH 7-8)	Invitrogen - Thermo Fisher Scientific	15513-047
Potassium Acetate	Merck	5044
IPTG	Sigma Aldrich	N/A
L(+)-Arabinose	Roth	5118.2
Phosphate-buffered Saline (PBS)	Thermo Fisher	N/A
Starch	Roth	4701.1
Iodine	Merck	N/A
Potassium Iodide (KI)	Merck	N/A
Chlorophenol red-β-D-galactopyranoside (CPRG)	Merck Sigma-Aldrich	99792-79-7
Ampicillin	Sigma Lifesciences	
Penicillin G Sodium	Sigma Lifesciences	P3032-100MU
Chloramphenicol	Sigma Lifesciences	N/A
Apramycin	Sigma Lifesciences	N/A
Kanamycin	Sigma Lifesciences	N/A
Sodium phosphate, monobasic	Sigma-Aldrich	N/A

Sodium Chloride	Honeywell - Fluka	3534976
Sodium hydroxide	Honeywell - Fluka	1310-73-2
Potassium sodium tartrate, tetrahydrate	Merck Sigma-Aldrich	1.08087.0500
3,5-Dinitrosalicylic acid	Sigma Life Sciences	D0550-25G
D-(+)-Maltose, monohydrate	Duchefa Biochemie	6363-53-7
Bovine Serum Albumin (BSA)	BioRad	N/A
Bradford Reagent (Coomassie Brilliant Blue G-250, phosphoric acid and methanol)	BioRad	N/A
EcoRI Restriction Enzyme	New England Biolabs	N/A
SpeI Restriction Enzyme	New England Biolabs	N/A
XbaI Restriction Enzyme	New England Biolabs	N/A
PstI Restriction Enzyme	New England Biolabs	N/A
T4 DNA Ligase	New England Biolabs	N/A
Q5 DNA Polymerase	New England Biolabs	N/A
Q5 Buffer	New England Biolabs	N/A
Pfu DNA Polymerase	New England Biolabs	N/A
Pfu Buffer	New England Biolabs	N/A
PCR DNA and Gel Band Purification Kit	GE Healthcare	illustra™ GFX™
Plasmid Purification Kit	BIOKÉ - Macherey-Nagel	NucleoSpin Plasmid Easy Pure

Supplementary Table 3: Oligonucleotides

Reagent or Resource	**Source**	**Identifier**

amyA-F-NcoI GATCCTCCATGGCGGCCCG TAATCCCACGCTGTTACA	This thesis	**amyA_NcoI_F(primer name)**: N-terminal Primer (with SecTag)
amyA-F-NdeI GATCCTCATATGGCCCGTAA TCCCACGCTGTTACA	This thesis	**amyA_NdeI_F**: N-terminal Primer (without SecTag)
amyA-R-XhoI GATATCCTCGAGCCAATCAC CTCTTCGATAACCCA	This thesis	**amyA_XhoI_R**: C-terminal Primer
lacZ-F-NcoI GATCCTCCATGGCGGCCAC CATGATTACGGATTCACT	This thesis	**LacZ_NcoI_F**: N-terminal Primer (with SecTag)
lacZ-F-NdeI GATCCTCATATGGCCACCAT GATTACGGATTCACT	This thesis	**LacZ_NdeI_F:** N-terminal Primer (without SecTag)
lacZ-R-XhoI GATATCCTCGAGCCTTTTTG ACACCAGACCAACTG	This thesis	**LacZ_XhoI_R:** C-terminal Primer
pET26b_1_fwd ctaacttacattaattgcgTTGCGCT CACTGCCCGCT	This thesis	N/A
pET26b_1_rev GAATTAATTCATGAGCGGAT ACATATTTGAATGTATTTAGA AAAATAAACAAATAGGGG	This thesis	N/A
CamR_fwd atccgctcatgaattaattcTTACGCC CCGCCCTGCCA	This thesis	N/A
CamR_rev aggggtgttATGGAGAAAAAAAT CACTGGATATACCACCGTTG ATATATCCC	This thesis	N/A
pET26b_2_fwd ttctccatAACACCCCTTGTATT ACTG	This thesis	N/A

pET26b_2_rev CGCAATTAATGTAAGTTAGC	This thesis	N/A

Supplementary Table 4: Recombinant DNA

Reagent or Resource	**Source**	**Identifier**
gapA	(*Registry of Standard Biological Parts*, n.d.)	Part: Bba_J100233
mCherry	Lab Stock	N/A
tdTomato	Lab Stock	N/A
pSB1A3 plasmid Amp^R	(*Registry of Standard Biological Parts*, n.d.)	Part: pSB1A3
pSB1C3 plasmid Cm^R	(*Registry of Standard Biological Parts*, n.d.)	Part: pSB1C3
pET26b(-) Kan^R plasmid	Prof. Erik Vijgenboom	NcoI site moved 6 bp upstream
pET26b(-) Cm^R plasmid	This thesis	N/A
amyA	(Kitagawa et al., 2005; *NBRP Ecoli Strain - ASKA Collection*, n.d.)	JW1912-AM
lacZ	(Kitagawa et al., 2005; *NBRP Ecoli Strain - ASKA Collection*, n.d.)	JW0335-AM
pCA24N plasmid	(*NBRP Ecoli Strain - ASKA Collection*, n.d.)	N/A
pET26b(-) amyA Cm^R	This thesis	A1_pET26_CmR
pET26b(-) pelB amyA Cm^R	This thesis	A2_pET26_CmR
pET26b(-) lacZ Cm^R	This thesis	L1_pET26_CmR
pET26b(-) pelB lacZ Cm^R	This thesis	L2_pET26_CmR

Supplementary Table 5: Instrument Details

Resource	**Source**	**Identifier**

Ultrasonic Homogeniser (Sonicator)	Badelin Sonopuls	4000 Series
Zeiss LSM AiryScan Confocal Microscope	Zeiss	N/A
Zeiss Benchtop Microscope	Zeiss	AX10
Refrigerated Centrifuge	Eppendorf	N/A
UV/Visible Spectrophotometer	Amersham Biosciences	Ultrospec 2100 pro
Micro-Plate Reader	Tecan	Spark
Scanner	Epson	Perfection V600 Photo
Multifuge 3 S-R	Heraeus	N/A
Nanodrop Spectrophotometer	Isogen Life Science	ND-1000
Gel Imager	Bio Rad	Gel Doc™ EZ Imager

Protocols

L-form *E. coli* Transformation

E. coli L-forms cells were transformed with pSB1-Cm^R and pET26b(-) Cm^R plasmids using protocols that were tweaked with every iteration to optimize the process (Supplementary Table: Iterative Changes to L-form *E. coli* Transformation Protocols). Attempt #15 was successful and the result could be replicated. The protocol:

1. Subculture *E. coli L-forms* cells and grow them overnight. Preculture cells into fresh media and grow to an OD600 of 0.2-0.3. This takes approximately 4-6 hours.
2. Aliquot 1ml of the culture into a 1.5ml microcentrifuge tube and spin down at 2700g for 3 min at room temperature.
3. Remove 700ul of supernatant and reduce the volume of the cells to 300ul.
4. Place on ice for 2 minutes.
5. Add plasmid DNA, approximately 1-2 ug.
6. Add 200ul 25% PEG6000 and 68ul 2.5M Magnesium Chloride, both filter-sterilized.
7. Incubate the mixture on ice for 30 minutes.
8. Heatshock the cells at 42°C for 30 seconds.
9. Place on ice for 2 minutes.

10. Add 400ul LPB (supplemented with Magnesium Chloride and PenG400)
11. Recover the cells at 30°C for 1 hour.
12. Prepare LPMA plates supplemented with magnesium chloride, horse serum, PenG, and other appropriate antibiotics. Plate cells with a cotton swab and/or decanting.
13. Incubate the cells at 30°C overnight.

Competent Cells Protocol (*TSS Competent E. Coli Preparation · Benchling*, n.d.)

Supplementary Table 6: TSS Buffer composition for a final volume of 20ml.

Component	Stock (M)	Amount	Unit
MgCl2	2	0.300	ml
DMSO	-	1	ml
PEG (3350 or 8000)	-	2	g
LB medium	to final volume	to final volume	-

Protocol

1. Inoculate 1mL of liquid medium (LB or SOC) with E. coli strain of choice in a 1.5mL PP tube (snap-cap) and culture overnight at 37°C with rotation.
2. Prepare 100mL of liquid medium (LB or SOC) in a sterile 250mL Erlenmeyer flask.
3. Seed the 1mL culture into the 250mL flask and incubate at 37°C with 225 rpm rotation.

-Use this time to prepare the TSS buffer

1. Cultivate until the optical density of the culture under 600nm wavelength is 0.3 to 0.4 ($0.3 \leq OD600 \leq 0.4$) (should take approximately 2-3h).

-TSS buffer should be chilled by this point.

1. Once the proper optical density has been achieved, transfer the 100mL of culture into 2x 50mL PP centrifuge tubes and centrifuge under 2,700xg for 10 min at 4°C.
2. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
3. Chill TSS-suspended cells on ice for 15 min. Prepare/label 100x 1.5mL PP tubes (snap-cap) during this time.
4. Distribute 100 µL of TSS-suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.

NOTE: 100 µL will be used per transformation.

1. Cells can be used immediately, or stored at -80°C.

LPB / LPMA Media Protocol

Supplementary Table 7: LPB Liquid Media with PenG for L-form *E. coli* growth

Component	Stock (M)	Amount	Unit
TSBS	-	200	ml
YEME	-	200	ml
PenG400	0.4g/ml	400	ul
Magnisium Chloride (Filter Sterilised)	2.5	4	ml

Supplementary Table 8: LPMA-Iberian Agar Media for plates

Component	Stock (M)	Amount	Unit
TSBS	-	100	ml
YEME	-	100	ml
Iberian Agar	-	?	g
Magnisium Chloride (Filter Sterilised)	2.5	2	ml
Horse Serum (Filter Sterilised)	?	10	ml

Boiling Prep Protocol for Plasmid Isolation ('Protocol – Plasmid Isolation by Boiling Method (Miniprep)', 2021)

1. Inoculate a single colony of E. coli with a sterile toothpick and place it in a 2 ml tube containing LB medium + selection antibiotic. Shake 200 rpm overnight at 37 °C.
2. Prepare STET buffer according to (*Yu:STET - OpenWetWare*, n.d.)
3. Prepare 10mg/ml lysozyme in lysis buffer.
4. Prepare TE Buffer with 10 mM Tris-HCl and 1 mM EDTA (pH 8.0)
5. Pipet 1400 µl medium in Eppendorf tube and centrifuge 1 min. Remove supernatant.
6. Resuspend the pellet in 100 µl STET buffer and incubate 5 min at room temperature.
7. Add 5 µl lysozyme solution and incubate 1 min at 95-100 °C.

8. Centrifuge 10 min at 13000 rpm and remove pellet.
9. Add 105 µl isopropanol (-20 °C) to the supernatant and vortex.
10. Centrifuge 10 min at 13000 rpm and remove supernatant.
11. Add 400 µl 70% ethanol to the pellet and vortex shortly. Centrifuge 5 min at 13000 rpm.
12. Remove supernatant completely and repeat the alcohol wash to remove excess proteins.
13. Dry pellet 2 min in Speedvac or 10 min at 65 °C.
14. Resuspend pellet in 50 µl TE buffer + 1 µl RNase. Incubate 20 min at 65 °C and vortex.

Protocol for DNA Isolation from Agarose Gels

1. Always use a **freshl**y prepared Agarose/TAE solution (old agarose reduces the recovery of the DNA significantly)
2. Cut out fragment (use long wavelength UV, 365 nm), max. 2 lanes of a mini gel per eppendorf tube
3. Add 200 µl water, crush agarose slice as good as possible
4. Add 500 µl phenol (saturated with 10 mM Tris pH 7-8), mix very well, incubate at -80°C for 10 min or longer
5. 10 min in eppendorf centrifuge (**not** a cooled centrifuge) at Room Temperature, max rpm.
6. Take the water layer and extract once with chloroform.
7. Take water layer, add 40 µl 3 M KAc pH 7.0 (~ 1/10 volume) + 1 µl glycogen (20 mg/mL) and ≥ 2 volumes EtOH, mix by inverting tube several times
8. Incubate overnight at -20°C
9. Next day, precipitate, a very small white pellet should be visible. Wash the pellet with 70% EtOH, dry pellet during 5 min at RT (just leave open Eppendorf tube on the bench)
10. Resuspend pellet in 15 µl sterile water, vortex and keep on ice for 30 minutes or longer. Vortex a couple of times to make sure pellet is completely resuspended.

Protocol for Touch-Down PCR (Korbie & Mattick, 2008)

Material	**Quantity**
DNA	1ul
Forward Primer	0.5ul
Reverse Primer	0.5ul

dNTPs	2ul
Pfu Buffer 10x	5ul
Pfu Polymerase	0.5ul
Milli Q water	39.25ul
DMSO	1.25ul
Total	**50ul**

Protocol:

Initial	95°C	5 min
Denature	95	0:30 min
Annealing	Touchdown from 65°C with –0.3°C	0:30 min
Extension	72°C	3:30 min
	Repeat 44x	
Final Extension	72°C	10 min

Protocol for Genomic DNA isolation using PCI (How to Use Phenol / Chloroform for DNA Purification - NL, n.d.)

1. Add one volume of phenol:chloroform:isoamyl alcohol (25:24:1) to your sample, and vortex or shake by hand thoroughly for approximately 20 seconds.
2. Centrifuge at room temperature for 5 minutes at 16,000 × g. Carefully remove the upper aqueous phase, and transfer the layer to a fresh tube. Be sure not to carry over any phenol during pipetting.
3. Add 1 ul Glycogen (20yg/ul), 7.5M NH4OAc (0.5 x volume of sample) and 100% ethanol (2.5 x volume of sample + NH4OAc) to the aqueous phase.
4. Place the tube at –20°C overnight to precipitate the DNA from the sample.
5. Centrifuge the sample at 4°C for 30 minutes at 16,000 × g to pellet the gDNA.
6. Carefully remove the supernatant without disturbing the gDNA pellet.
7. Add 150 µL of 70% ethanol. Centrifuge the sample at 4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant.
8. Repeat Step 3 once. Remove as much of the remaining ethanol as possible.
9. Dry the gDNA pellet at room temperature for 5–10 minutes.
10. Resuspend the gDNA pellet in 50 µL of MilliQ water..

Protocol for Overexpressing Soluble Proteins in *E. coli*

1. Prepare 20% (w/v) Arabinose and a 0.5M stock solution of IPTG.
2. Transform the expression vector containing the ORF of your POI into your E. coli expression strain, plate cells on selective LB agar.
3. On the afternoon of the next day, pick a single colony and use it to inoculate a small preculture (e.g., 20 ml LB + 20 µl 1000x antibiotic stock solution). Grow this culture overnight at 37 °C and 200 rpm.
4. On the next morning, use this preculture to inoculate the expression culture in a ratio of 1:100 (e.g., 5 ml preculture + 500 ml LB + 500 µl 1000x antibiotic stock solution). Incubate the expression culture at 37 °C and rapid shaking (e.g., 150 rpm; but the shaking speed depends on the size of your expression culture; ensure fast shaking, but make sure that the medium cannot splash out of the expression flask).
5. Grow the expression culture until an OD600 of 0.6-0.7. Normally, this should take approximately 2.5 – 3.5 h. For L-form *E. coli* protein induction, grow the expression culture to OD600 of 0.2-0.3 (unpublished data). Typically this takes between 5-6 hours.
6. Withdraw a 1 ml sample from the expression culture and pipet it into an Eppendorf tube, which you tape to the side of the flask (= uninduced culture sample = UI).
7. Induce protein expression by adding IPTG at a final concentration of 0.5 mM to the culture. *in the case of BL21 AI, add arabinose to a final concentration of 0.2%
8. Keep on growing the expression culture.
    a. Default option 1: growth at 37 °C for four h.
    b. Default option 2: growth at 18 °C until the next morning (ca. 18 h).
9. Before harvesting the cells, withdraw a 1 ml sample from the expression culture and pipet it into an Eppendorf tube (= induced culture sample = I). Remove the UI sample from the culture flask. Centrifuge both samples at 11,000 g, 4 °C, 1 min, and decant the supernatant. Store the UI, and I samples in the freezer for subsequent protein expression analysis via SDS-PAGE.
10. Harvest the cells by centrifugation at 4 °C in tubes of a suitable size. Default parameters for centrifugation are 6,000 g, 15 min.
11. Decant the supernatant (= spent expression medium) and transfer the cell pellet into a suitable vial, such as a 50 ml Falcon tube. Make sure to keep the cell pellet on ice!
12. The cell pellet can be used directly for subsequent protein purification. Otherwise, it should be frozen in liquid nitrogen and stored at -20 °C or -80 °C.
    a. Option 1: Freeze cell pellet directly and resuspend in a suitable buffer upon thawing.

    b. Option 2: Resuspend cell pellet in a suitable buffer and then freeze the cell suspension.
13. Plan the next steps, which are cell lysis and purification of your protein of choice

Starch Agar - Iodine Assay Protocol

1. Prepare and autoclave 1% Starch-LB Agar and pour 50ml plates with the appropriate antibiotics.
2. Prepare Gram's Iodine stain with 1g Iodine and 2g Potassium Iodide in 300ml distilled water.
3. Overexpress proteins in the desired strain of *E. coli* according to the protocol in this thesis.
4. Spot 5ul of the live cell cultures on a starch agar plate and incubate at 37°C for 18 hours.
5. Spin down 10ml aliquots in a cooled centrifuge for 20 min at 4000g, 4°C.
6. Spot 5ul of the supernatant on the starch-agar plates from different samples and incubate the plate at 37°C for 4-18 hours.
7. Discard the remaining supernatant and resuspend the cell pellet in 700ul 1x PBS.
8. Sonicate each sample 3 times at 10% amplitude using an MS72 probe for 20 seconds with 1-second pauses. Keep the samples on ice between each sonication to ensure that samples do not overheat.
9. Spin down the sonicated samples at 16,000g, 4°C for 30 minutes. The proteins accumulate in the PBS.
10. Spot 5ul of the supernatant of the sonicated samples on starch agar plates and incubate for 4-18 hours at 37°C.
11. Stain all starch agar plates after incubation with Gram's Iodine for 30-60 seconds. Decant and discard the excess stain.
12. Image the plates with a camera placed at a standardised height using a clamp.
13. Perform halo size analysis on the images using ImageJ.

DNS Assay

Prepare reagents according to specifications in (Merck, n.d.)

1. Alpha Amylase assay
    a. Overexpress proteins in the desired strain of *E. coli* according to the protocol in this thesis.
    b. Spin down 10ml aliquots in a cooled centrifuge for 20 min at 4000g, 4°C.
    c. Discard the remaining supernatant and resuspend the cell pellet in 700ul 1x PBS.

d. Sonicate each sample 3 times at 10% amplitude using an MS72 probe for 20 seconds with 1-second pauses. Keep the samples on ice between each sonication to ensure that samples do not overheat.
e. Spin down the sonicated samples at 16,000g, 4°C for 30 minutes. The proteins accumulate in the PBS.
f. Add 1 ml of the standard starch solution to each sample vial. Leave one out for the blank sample.
g. Mix by swirling and equilibrate to 20 °C. Then add 0.4ml of the sonicated alpha amylase sample from each E. coli strain.
h. Mix by swirling and incubate for exactly 3.0 minutes at 20 °C. Then add 0.5ml of the Color Reagent
i. Cover containers with a vented cap and place in a boiling water bath for exactly 15 minutes.
j. Cool solutions on ice to room temperature.
k. Then add 9ml of sterilized Milli Q water to each sample.
l. Mix by inversion. Blank a suitable spectrophotometer against air at 540 nm and record the A540 for the Samples and Sample Blank.

2. Standard Curve Preparation
    a. Prepare a standard curve by pipetting (in mL) the following reagents into suitable containers:

Supplementary Table 9a: DNS Assay Standard curve preparation

Reagent	**STD1**	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD BLK**
0.2% (w/v) Maltose Standard	0.05	0.20	0.40	0.60	0.80	1.00	2.00	–
Ultrapure water	1.95	1.80	1.60	1.40	1.20	1.00	–	2.00
Color Reagent	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

    b. Cover containers with a vented cap and place in a boiling water bath for *exactly* 15 minutes.
    c. Remove containers from the boiling water bath. Cool solutions on ice to room temperature.
    d. Then add (in mL):

Supplementary Table 9b: DNS assay standard curve preparation

Reagent	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD BLK
Ultrapure water	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00

e. Mix by inversion. Blank a suitable spectrophotometer against air at 540 nm and record the A_{540} for the *Standards* and *Standard Blank.*

3. Calculations
   a. Determine the ΔA_{540} of each *Standard* vs. the *Standard Blank*.
      ΔA_{540} (Standard) = A_{540} (Standard) – A_{540} (Standard Blank)
   b. Prepare a standard curve by plotting the ΔA_{540} of the standards vs. mg of maltose using linear regression.
   c. Determine the ΔA_{540} of each *Sample* vs. the *Sample Blank*.
      ΔA_{540} (Sample) = A_{540} (Sample) – A_{540} (Sample Blank)
   d. Determine the mg of Maltose released using the standard curve.

Eqn1: $Units/ml\ enzyme\ =\ [(mg\ of\ maltose\ released)\ *\ (df)]\ /\ [(mL\ of\ enzyme)]$

where:
df = dilution factor
mL enzyme = mL of Sample added in step 1(g).

Eqn2: $Units/mg\ solid\ =\ [units/(ml\ enzyme)]\ /\ [(mg\ solid)/(ml\ enzyme)]$

CPRG Agar Assay Protocol

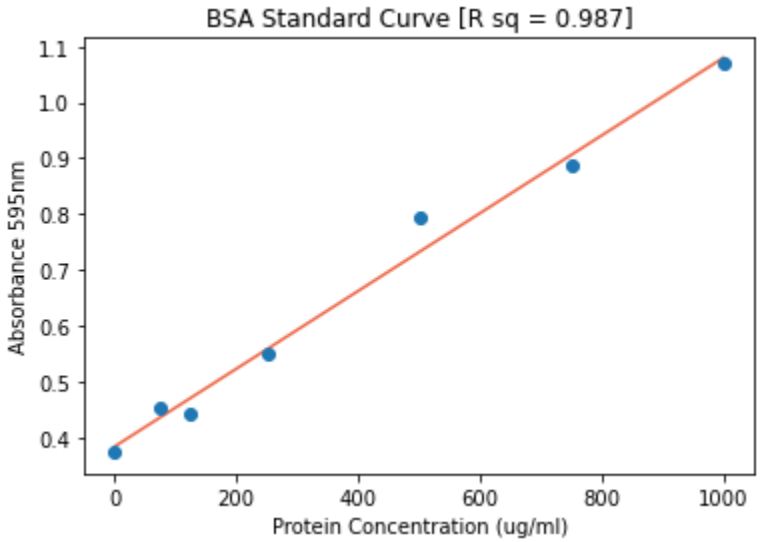
1. Prepare LB agar supplemented with 20ug/ml CPRG and 50ml plates with the appropriate antibiotics.
2. Overexpress proteins in the desired strain of *E. coli* according to the protocol in this thesis.
3. Spot 5ul of the live cell cultures on a CPRG agar plate and incubate at 37°C for 18 hours.
4. Spin down 10ml aliquots in a cooled centrifuge for 20 min at 4000g, 4°C.
5. Spot 5ul of the supernatant on the CPRG-agar plates from different samples and incubate the plate at 37°C for 4-18 hours.
6. Discard the remaining supernatant and resuspend the cell pellet in 700ul 1x PBS.

7. Sonicate each sample 3 times at 10% amplitude using an MS72 probe for 20 seconds with 1-second pauses. Keep the samples on ice between each sonication to ensure that samples do not overheat.
8. Spin down the sonicated samples at 16,000g, 4°C for 30 minutes. The proteins accumulate in the PBS.
9. Spot 5ul of the supernatant of the sonicated samples on CPRG agar plates and incubate for 4-18 hours at 37°C.
10. Image the plates with a camera placed at a standardized height using a clamp.
11. Perform halo size analysis on the images using ImageJ.

Bradford Assay

The goal is to estimate the total amount of protein present in each *E. coli* strain, both in the supernatant and sonicated samples.

1. Thaw on ice the supernatant and sonicated *E. coli* samples prepared in the Starch Agar and CPRG Agar assay.
2. Make a 10x and 100x dilution for each sonicated sample and a 10x dilution for each supernatant sample in PBS.
3. Prepare BSA dilutions for standardization.
4. Load Bradford Reagent into each well of a 96-well plate.
5. Load 5ul of each sample into individual wells of the 96-well plate.
6. Measure the absorbance of each sample at 595nm in a Tecan spectrophotometer.
7. Calculate the standard curve for BSA using linear regression. Calculate the amount of protein with reference to the standard curve (Supplementary Figure 1).



Supplementary Figure 1: BSA standard curve for the Bradford assay.