AN INVESTIGATION OF A TEMPERATURE-SENSITIVE INTEIN WITH REGARDS TO ITS USE AS METABOLIC CONTROL ELEMENT FOR BIOPROCESS

ENHANCEMENT

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Abstract

Bioprocesses based on metabolically engineered microbes have become tremendously important in recent decades as a platform for the synthesis of complex molecules. Substantial research effort has been devoted to the improvement of microbial strains involved, and while this has enhanced some metrics of strain performance dramatically, namely product yield with respect to substrate and biomass, achieving similar results with other aspects has remained elusive. Improving productivity, the rate at which a modified strain can synthesize a product of interest, in particular has presented an engineering challenge despite its obvious value to the economics of a process and has typically only been done through bioprocess optimization. A strategy that could yield the desired result is strain engineering to better integrate with the bioprocess context in which it is used. The work described in this thesis has sought to achieve that goal by providing a method to the operating engineer to dynamically control the induction of genes associated with product formation. More specifically, a T7 RNA polymerase was modified by the insertion of a mutant variant of the S cerevisiae Vacuolar membrane ATPase (VMA) intein. This mutant intein will only splice out of its host only under conditions of reduced temperature, which in effect makes the polymerase active only after a temperature shift from 37° C to 18° C degrees. This creates a strict demarcation between biomass accumulation and product synthesis, only allowing this transition to be made at an optimal point during fermentation, as chosen by the operating engineer. Using lycopene biosynthesis as a case study and applying this approach, it was found that a productivity improvement of approximately 15% over an uncontrolled strain was attained. It was also found that a remarkable degree of control stringency was conferred upon the system, with no premature product synthesis detected under any condition investigated. These results are expanded upon to generate a series of simple mathematical models, with the aim of describing how such a dynamic metabolic control element might be expected to perform in a more generalized context, and to provide a means by which to more quantitatively assess the strain's performance.

Lay Summary

In recent decades, the production of fine and commodity chemicals has increasingly been done through the use of metabolically engineered microbes. The aim of this thesis is to engineer such a microbe, and to demonstrate how its productivity can be improved by including a generalizable enzymatic switch that lets the operator turn on the microbe's ability to produce the chemical lycopene, used here as a test case, when it is most efficient to do so. The input signal required to activate the switch is a drop in temperature from 37° C to 18° C, chosen because it has previously been shown to improve microbe-based lycopene production, and because temperature is a parameter over which the operator has a high level of control. Other inputs could be used to create this improvement, if the system was engineered appropriately, and some of these possibilities are theoretically explored as well.

Preface

This thesis, entitled "An Investigation of a Temperature-Sensitive Intein with regards to its Use as a Metabolic Control Element for Bioprocess Enhancement" presents the research that I performed during the course of my MASc, and was identified, initiated, and supervised by Professor Vikramaditya G. Yadav. A version of part of this study, namely the methods used, and some the data in sections 4.2, 4.3, and 4.4, was published previously (Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.). For this paper, I conducted experiments and gathered data. The numerical model was developed, and the analysis performed by Dr. Yadav and I. I prepared the first draft of the paper which was subsequently revised by Dr. Yadav.

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1. Introduction

The bacterium *E. coli* has served as the most popular platform of protein expression since even before the inception of molecular biology as a distinct discipline. *E. coli* serves as a robust, fast-growing host, requires only inexpensive media, has been shown extensively to be able to express a wide variety of proteins, and is the subject of a substantial body of scholarship which provides not only a level of fundamental understanding exceeding that of perhaps any other organism, but describes numerous examples of its exceptional tractability to genetic manipulation^{1,2,3}. In recent decades its use has moved beyond the laboratory, and now plays a central role in the biotechnology industry, where it produces protein therapeutics and fine chemicals valued in the tens of billions of dollars annually^{4,5}.

Although many factors affect the success and performance of a bioprocess, there are three general metrics that have emerged as the standard by which to judge the effectiveness of an engineered strain as a biocatalyst within this context: yield, referring to how efficiently a strain converts substrate to product, titer, the final concentration of product the strain is able to produce at the end of the fermentation period, and productivity, a measure of the capacity of a strain to catalyze a reaction, and is generally given in terms of the change in product per mass of dry cell weight with respect to time^{6,7}. While fermentation conditions and strategy have a dramatic impact on these metrics, particularly with respect to productivity, extensive work has gone toward improvement of the strains themselves through genetic engineering during upstream phase of the bioprocess. Efforts to improve titer have generally revolved around the selection of strains to relate to high concentrations of product and preventing the formation of harmful metabolic byproducts⁸. Improvements in yield have generally been achieved by diverting as much metabolic flux into the pathway of interest as possible, while productivity improvements

typically involve a similar strategy, but with a focus on increasing the overall magnitude of flux through a particular pathway 9,10 . However, the changes applied to a strain with the aim of improving any of these metrics do not exist in isolation, and a major challenge central to metabolic engineering is that any metabolic change implemented to improve any single one of these metrics will inevitably harm the other two^{11,12}. Diverting metabolic flux to improve yield, for example, will result in better conversion to the product of interest, but will reduce the transfer of flux to biomass, and thereby reducing the productivity of the process as a whole. Those efforts which have met with the greatest success use a more holistic approach to metabolic engineering, leveraging an improved understanding of intracellular dynamics and metabolic analysis software to identify those alterations to the strain resulting in optimal performance, and which fermentation strategy is best suited to the unique demands of the improved strain^{13,14}. One such successful strategy is the delineation of fermentation into at least two distinct regimes: a biomass accumulation phase, and a subsequent product generation phase¹⁵. However, while the distinct properties possessed by E coli's growth phases have long been understood, few tools exist to induce a change in cellular expression in an on-line bioprocess.

It is surprising, then, that relatively few advancements in the dynamic control of protein expression have so far been described in the literature. Currently, the most popular approaches are either the addition of small molecule inducers to the bacterial culture, most prominently Isopropyl β -D-1-thiogalactopyranoside (IPTG), at the point during growth at which expression is desired, or constitutive expression, where a protein is expressed by the cell at all points during growth¹⁶. However, both of these approaches have substantial issues for use at industrial scale, with IPTG and similar molecules being far too expensive to be an economical solution, while constitutively expressing a protein will cause a cell to divert resources away from biomass

production while a culture's density is still low, thereby lowering the growth rate, and decreasing the efficiency of a process overall¹⁷.

Another commonly used element for protein expression in *E. coli* is the T7 RNA polymerase. T7 polymerase is far more active than the cell's native RNA polymerase, and genes whose transcription is governed by this enzyme can have their encoded protein accounting for as much as 40% of the cell's dry weight¹⁸. The high activity of T7 polymerase is the source of its principal disadvantage as well. Transcription of most genes is inherently "leaky", that is, low-level expression can still occur even when the gene is uninduced. With a highly active enzyme like T7 polymerase, even a few copies expressed per cell can in turn result in non-trivial levels of expression of whatever gene is under the control of T7 polymerase¹⁹. This results in poor delineation of the fermentation growth phases, as the rate of biomass accumulation in the first phase is slowed due to the baseline expression of genes controlled by the T7 promoter. This can also pose a serious problem for researchers investigating toxic proteins, as the level of expression even when uninduced may be adequate to prevent growth of the host strain²⁰.

Inteins are segments of protein capable of excising themselves from within another protein and ligating that protein's flanking regions together with, in many cases, no deleterious effect on normal structure and function after excision²¹. The vacuolar membrane ATPase (VMA) intein of *S. cerevisiae* was the first intein identified and remains the most thoroughly studied. Zeidler et al. (2004) generated a library of temperature-sensitive VMA inteins, meaning that only below a certain permissive temperature will they excise themselves²². T7 RNA polymerase has previously been shown to have a site at which an intein can be inserted into and successfully excised from, and it is this previously identified insertion site which is used here²³.

The goal of this work is to describe a method for using a T7 RNA polymerase containing a temperature-sensitive intein as a novel approach for improving upon common methods of

protein expression control. Three versions of an intein-containing T7 polymerase were constructed to compare with the wild type enzyme: a dead intein, whose capacity for selfexcision has been removed, thereby rendering the polymerase inactive under all conditions, a wild-type intein, which will rapidly self-excise after translation under all conditions, and a temperature-sensitive intein, which should excise itself only at the lower permissive temperature of 18° C (Figure 1.1). These constructs were subsequently tested by transforming them into strains expressing GFP and Lycopene synthesis genes under the control of a T7 promoter. A dramatic improvement in the stringency of expression control of these genes was observed, and an improvement in the productivity of lycopene relative to strains expressing wild-type T7 polymerase. These results demonstrate the potential of dynamic protein expression control as an approach to improve bioprocess performance.



Figure 1.1 All T7 RNA polymerase constructs used in this work. Strains containing these constructs will be referred to as pRNAP, for wild-type T7 RNA polymerase, pRNAP-WT, for the T7 RNA polymerase interrupted by a wild-type VMA intein, pRNAP-TS, for the construct interrupted by an intein which will only excise at 18 °C, and pRNAP-Dead, containing an intein unable to excise under any condition. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.

2. Literature Review and Background

2.1 The Intein

While inteins have been known to science for longer, the first sequence was published almost exactly 30 years ago²⁴. The science of molecular genetics was still in its infancy, and as such, it was not immediately apparent why the gene encoding Saccharomyces cerevisiae Sce VMA1 vacuolar ATPase was so much larger than its bacterial and human counterparts. Only two years after this, several research groups showed that a significant fraction of this sequence was not actually present in the mature enzyme. This represented a significant challenge to the orthodox paradigm of protein expression held at the time, as an internal gene domain being removed by protein splicing, rather than RNA splicing, had never previously been demonstrated. The resulting fragments, both of which were found to form stably folded proteins in their separated state, were dubbed the extein, from the host protein, and the intein, the interrupting protein^{25,26,27}.

All attempts to disprove this new paradigm failed, and a series of experiments in subsequent years definitively established protein splicing as a new method of gene expression. Several important mechanistic insights were gleaned from this period, namely that splicing can only occur when a branched intermediate forms at the N-terminal junction of the intein and extein fragments, and that in at least some demonstrated cases, splicing can be controlled^{28,29,30}.

The only necessary condition for proper splicing to occur is that the intein domain is allowed to fold correctly, so as to align the relevant nucleophilic residues involved in the splicing reaction, and those residues involved in the reaction. In the VMA intein those necessary residues were found to be a cysteine as the first residue at the N-terminal, and an asparagine and cysteine at the 454 and 455 position, respectively. However, there is some degree of variation in the identity of those residues across intein class, and particular inteins within the same class. No, additional chaperone, cofactor, or even energy source is required to allow this reaction to proceed^{31,32}.

Three broad classes of inteins are currently recognized in the literature, with inteins being assigned based on conserved sequence motifs and similarities in splicing mechanism. The most common class one, of which the VMA Sce intein is an example, has a splicing mechanism consisting of a.) an acyl rearrangement to convert the N-terminal splice site peptide bond from an amide to a thioester, b.) a transthioesterification to form a branched intermediate, c.) resolution of the branched intermediate via the cyclization of the Asn residue, resulting in cleavage at the C-terminal junction and d.) an amide bond is formed between the now ligated extein segments after a second acyl shift³³⁻³⁶ (Figure 2.1).

Since the initial discovery of the VMA intein, a surprising variety of inteins have been subsequently shown to exist. The typical intein is a large, multi-function, multi-domain, stably-folding cytosolic protein, usually consisting of several splicing-related domains, and a large homing endonuclease which functions essentially as a selective marker. In contrast, examples of inteins under 200 residues in length have been described in the literature, known as "mini-inteins." These inteins lack the endonuclease domain and function associated with their larger cousins, and as such have played a vital role in research working to define the minimum necessary intein splicing domain³⁷⁻³⁹.

One remarkable feature seen in some types of inteins is their capacity to splice even when split, that is, split inteins can be expressed in two or more discrete fragments in trans within the host cell, and splicing can still occur. Several examples of naturally occurring split inteins have

been identified. These include the Ssp DnaE intein, which was identified interrupting the DNAE catalytic subunit of DNA polymerase in the Synechocystis and Nostoc cyanobacterial strains, and has found great and varied utility in biotechnological applications⁴⁰⁻⁴².



Figure 2.1 The mechanism of class 1 intein splicing. If splicing is able to proceed, two discrete proteins result: the spliced intein, now functioning as a homing endonuclease in the case of the VMA Sce intein, and the religated N- and C-extein protein, indistinguishable in structure and function from an isoform which has not previously hosted an intein. From: Rosa, Lucia De, et al. "Semi-Synthesis of Labeled Proteins for Spectroscopic Applications." *Molecules*, vol. 18, no. 1, Feb. 2013, pp. 440–465., doi:10.3390/molecules18010440.

2.2 The Origin and Function of Inteins

"Homing" when used as a descriptor of a genetic element, refers to the ability of that element to duplicate and transfer itself to a particular allele not already containing that element. It therefore serves as a mechanism by which such gene elements can in effect "reproduce" outside the normal rules of reproduction and inheritance observed by their hosts, and rapidly propagate through a host population. While this homing activity was first identified as a property of certain introns that encode homing endonucleases, many inteins, including the VMA intein, use this mechanism to spread as well. When an intein- or intron- free allele is present in the same cell as an allele of the gene which contains a homing endonuclease-encoding intein or intron, the endonuclease will cleave the unmodified sequence, causing a double-stranded break in the genome. This either kills the cell or causes the propagation of the intein/intron by homologous repair mechanisms at the broken site⁴³⁻⁴⁶.

The existence of inteins is most easily explained if they are viewed as what is referred to as selfish genetic elements. While what genes can and cannot be considered "selfish" is a subject of some debate, a generally accepted definition is that selfish genes are to some degree parasitic, contributing primarily to its own success even at the expense of its host organism's fitness. In the case of inteins, the homing activity which allows them to propagate directly harms their host, causing DNA damage and consuming metabolic resources^{47,48}.

Homing endonucleases are highly specific in the gene sequences they target. A typical homing endonuclease recognizes a site of between 14-40 DNA base pairs, and although all tend to demonstrate some degree of promiscuity in the sequence they recognize, this is still more than long enough to ensure only the intended sequence will be recognized in a single genome. The Sce

VMA intein, for example, has a recognition site of 31 bp in length, but only 9 of these will result in the abolition of nuclease activity if mutated in the target^{49,50}.

It is perhaps unsurprising then that inteins are typically found within highly conserved regions of proteins that are, in taxonomic terms, themselves highly conserved. These proteins are generally associated with functions critical to the cell, such as DNA synthesis and energy metabolism. It remains a subject of some controversy exactly why inteins are found at the particular integration sites seen. However, there are a number of explanations generally agreed upon. Since the homing mechanism of intein replication is dependent on the cell's native DNA repair infrastructure repairing the double-stranded break, it is logical that the most common class of intein host protein would be those proteins involved in these functions, as it ensures if the endonuclease is present in the cell the proteins required for DNA repair will be as well. Inteins are found in conserved regions of proteins because if the intein is deleted imprecisely, the function of the host protein is likely to be completely abolished, and therefore making an unlikely perfect deletion of the intein the only scenario which won't be selected against by the host organism⁵¹⁻⁵⁴.

The hypothesis that inteins originated and spread through their role as parasitic genetic elements provides an adequate explanation for the many large inteins which contain homing endonucleases. However, it ignores the existence of mini and split inteins, which have lost all enzymatic functionality except for the ability to splice themselves from their host protein. To fully explain the origins of inteins requires reference to what is referred to as the homing cycle. This cycle is broken into four steps, beginning with 1.) the initial invasion of a host protein at a suitable site by an intein containing a homing endonuclease. Through endonuclease-based cleavage and homing, the intein becomes established throughout the host population and is stably inherited. Since the endonuclease is a large and costly enzyme to the host organism, and since the

precise deletion of the intein is a highly unlikely event, this leads to 2.) the gradual degradation of the endonuclease domain while maintaining the self-excision domains allowing the host protein to function. Eventually, the degraded intein is removed entirely from its insertion site by the host's deletion bias pressure, which constitutes step 3.). Finally, step 1.) is repeated as inteins with functional endonuclease domains reinvade the now wild type cell lines by horizontal gene transfer from subpopulations of the host species which have continued to maintain them. Mini and naturally split inteins are thought to be derived from inteins at step 2.) of this cycle. However, it is thought that in addition to simply becoming smaller to minimize their detrimental impact on the host cell, those mini-inteins that have become ubiquitous throughout large and widely distributed species, as many of the most thoroughly studied mini and split inteins are, have actually evolved to confer some advantage upon their host. The naturally split Npu intein, for example, may allow the host to utilize more complex and fine-tuned logic in its gene expression by expressing fragments of a single protein in trans from different regions and promoters in its genome. Any potential positive role for these inteins remains controversial however, and no conclusive evidence exists to support this concept⁵⁵⁻⁵⁸.

2.3 Inteins in Biotechnology

Interins represent a promising tool for biotechnology, and a variety of applications have been demonstrated. Proteins that are normally far too toxic for reliable expression can be interrupted by an intein to temporarily abolish this toxicity. This can be done either with a conditionally splicing intein, and activated later, in vitro, or a split intein recombined in vitro after two separate fermentations. Split inteins can be used to aid nuclear magnetic resonance analysis of proteins as well. By producing a protein in discrete fragments over multiple

fermentations and including isotopically labelled growth substrate to the media of only one, the researcher can choose what region of the protein is labelled once reconstituted in vitro^{59,60}.

The first commercial applications of inteins were as aids in the purification of recombinant protein, and this is the role in which they still find the most prominent use today. Small to medium scale protein purification is commonly been based on the incorporation of affinity tags, such as histidine motifs or the maltose-binding protein, and using these tags to bind the fusion protein to chromatography column with affinity to that tag. This method has significant limitations however, including expense of the eluent buffers, the maximum throughput of the process as a whole, and the functionality, stability, and if meant as a therapeutic, immunogenicity of the target protein⁶¹.

Soon after the discovery of the VMA intein, the first advances in conditional intein splicing were made. By making certain mutations to the active site of the splicing domains at either the N or C terminus of the intein gene, it was found that complete splicing could be prevented from occurring unless the intein was exposed to a reducing agent. By inserting a purification tag into the intein's endonuclease domain, and then inserting the intein into an appropriate site in the protein of interest, the researchers created what is in effect a conditionally self-excising purification tag. This system was subsequently commercialized by New England Biolabs, and purification kits based on this concept are still available under the IMPACT trade name. However, this system does not solve the issues with throughput associated with column-based purification, dramatically increases the time required for excision by the intein, requiring an overnight incubation, and the addition of reducing agents renders this system unsuitable for many proteins⁶².

A much improved intein-based purification system is derived from the Δ I-CM miniintein. After random mutation and screening, a variant of this intein which splices only after a

slight decrease in Ph was identified. This intein variant completes its excision reaction over a much shorter time frame, and the requirement for only a Ph shift makes the reaction much cheaper to induce, and results in a much gentler chemical environment for the protein of interest than reducing agents⁶³.

Recently, the VMA intein was artificially split, and each fragment fused to one of the proteins FKBP12 and FRB, replacing the endonuclease domain. These proteins dimerize in the presence of rapamycin, and so when this molecule is added to the solution it causes the splicing domains of the intein to be brought into close proximity to each other. If the intein fragment end distal to the rapamycin-binding domain is fused to another protein, they can they undergo their splicing reaction as normal, in effect creating a small molecule-induced splicing system. This approach has been demonstrated with other dimerizing domains as well. Replacing FRB/FKB12 proteins with coiled coil domains creates a system where splicing can only occur after cleavage of the coiled coil allows the required domains to interact, making it therefore dependent on the expression of the cognate protease. Several inteins have been engineered for conditional splicing in response to certain wavelengths of light. The first inteins to demonstrate this property were engineered by photocaging the protein with a light-cleavable moiety. Subsequent efforts to produce light-responsive systems utilized split inteins, in combination with light dimerizing domains, including LOV2 proteins combined with the DnaE intein, and Phycocyanin domains combined with VMA Sce. It should be noted, however, that creating these kinds of fusion inteins always greatly affects splicing performance, with the reaction being slowed dramatically, and often require relatively low temperatures to proceed at all. Therefore, these systems are of limited utility for many applications, despite their versatility⁶⁴⁻⁶⁸.

Although Inteins have been applied in many remarkable ways in biotech, it is the engineering of inteins to splice conditionally, as demonstrated in the prior examples, that has

produced some of the most valuable advancements in the field, and the aspect of inteins most relevant to this work. In order for an intein to be used in controlling a cellular process, and to be at all useful for most applications, it must be capable of splicing conditionally. While a few inteins possess this property naturally, such as a responsiveness to temperature in thermophile-derived sequences, most are the result of extensive engineering work, and inteins with many different splicing triggers have been demonstrated. The Sce VMA intein in particular, as one of the first identified and most thoroughly studied inteins, has been altered to splice conditionally in a variety of ways⁶⁹.

One area of conditional splicing research that has yielded especially valuable results is that of temperature-sensitive inteins. Zeidler et al (2004) generated a library of temperature sensitive VMA Sce inteins through random mutation. By inserting these inteins into a Gal80 and Gal 4 transcription factor, the researchers were able to use a temperature downshift to induce expression of target genes in both S cerevisiae and D melanogaster. More recently, the inteins in this library were extensively mutagenized to broaden the range of effective temperatures at which these inteins could be triggered to splice. The researchers inserted the inteins into an enzyme which allowed the host yeast strain to complement an auxotrophic phenotype upon splicing, thereby allowing only those cells containing an intein with the desired qualities to grow. Beyond just generating a larger library of temperature-sensitive inteins however, the researchers conducted several experiments which were arguably more valuable for informing works such as this one, which seek to use these inteins as an *in vivo* control element. A selection of inteins deemed promising based on results from earlier in the work were inserted into several other proteins as well, including the LacZ enzyme. First, this allowed the researchers to demonstrate, if only in approximate terms, to what degree interrupting an enzyme with one of these temperaturesensitive inteins will affect its performance. Secondly, engineered inteins often display a strong

dependence on context, with kinetics and even temperature of folding varying widely depending on what protein they are inserted into. One particular intein stood out on both these metrics, with only a modest decline in splicing time relative to wild-type, and with little difference seen between the different proteins tested. It is this high-performing intein used in the present work, and all variations of T7 polymerase denoted as "temperature sensitive" are interrupted with it^{70,71}.

2.4 The MEP and MVA Isoprenoid Pathways

Isoprenoids, also sometimes referred to as terpenoids, are one of the largest groups of natural compounds known to science, and members of this group demonstrate an enormous degree of structural diversity and function in biological systems⁷². Demonstrated roles for these molecules include cell-wall biosynthesis, post-transcriptional and translational modifications, the electron transport chain and photosynthesis, intra- and extracellular signaling, and even sometimes play a direct role as a chemical defense mechanism. Several of their physical properties, including high vapor pressure, and relatively low molecular weights, make them ideal candidates for such roles. Isoprenoids are produced by members of every kingdom of life, and a large fraction of secondary metabolites produced by microbes and plants are grouped with these molecules⁷³⁻⁷⁶.

Although many different isoprenoids have found uses in human society for thousands of years, including as medicine, dyes, or flavor compounds, very little about their properties or structure was understood until the chemical revolution of the late 19th century. In the mid-20th century, a newfound understanding of biochemistry led to an appreciation of the importance of terpenoids, in particular the cholesterols, in human biology, which led in turn to the characterization of the metabolic pathways leading to their synthesis. Subsequent experiments

with isotopically-labelled acetate demonstrated that all isoprenoid synthesis proceeded through a few fundamental molecular building blocks, namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Work by several other research groups reconstructed the corresponding sequence of biochemical reactions, which upon its reconstruction and characterization was named the Mevalonate pathway, after a key intermediate, mevalonic acid (MVA)⁷⁷⁻⁸⁰.

The first dedicated steps of the mevalonate pathway begin with two transferase reactions, each involving the usage of a coenzyme A (CoA) moiety. The condensation of two molecules of acetyl-CoA is catalyzed by an acetoacetyl-CoA thiolase and then followed by a synthasemediated aldol reaction between the ketothioester and a third acetyl-CoA molecule moiety. This sequence yields the first six-carbon intermediate, HMG-CoA. Both enzyme catalysts form covalent adducts with their respective reaction intermediates and are cofactor-independent. Release of the cofactor by HMG-CoA synthase requires a water molecule for hydrolysis. An NADPH-dependent reduction leads to the formation of MVA and the release of CoA. The reaction is catalyzed by HMG-CoA reductase (HMGR) and follows the formation of a mevaldehyde intermediate. Protonation of the aldehyde moiety leads to the formation of (R)mevalonate. The steps following mevalonate production require two ATP-dependent phosphorylations at the terminal mevalonate hydroxyl group. These result in the formation of mevalonate-5-phosphate and mevalonate-5-diphosphate, catalyzed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK). PMK transfers a terminal phosphate from ATP. The final step of the MVA pathway is a dehydration and decarboxylation catalyzed by mevalonate diphosphate decarboxylase (MDD) resulting in isopentyl pyrophosphate (IPP)⁸¹⁻⁸⁵ (Figure 2.2).

Since its initial discovery, the MVA pathway has been discovered to be the sole or primary route to terpenoid molecule synthesis in many different classes of organisms, including

not only humans, but plants fungi, and many archaea and bacteria as well. Some deviations in the reaction pathway described above were identified since its initial discovery, in particular the existence of an alternate synthesis route to IPP through phosphomevalonate decarboxylase and isopentenyl phosphate kinase, seen in some plants and archaea. These were considered minor, peripheral additions to what was considered an almost universally conserved pathway and did little to disrupt the acceptance of the MVA pathway as the sole source of biological isoprenoid synthesis^{86,87}.

In the 1990's a series of further isotopic-exchange experiments on a wider variety of model organisms generated results which were inconsistent with the biochemistry of the MVA pathway. Upon closer investigation, it became clear that not only was this pathway not the only route to isoprenoid biosynthesis, but that in many organisms it was completely absent, despite the near ubiquity of isoprenoid compounds and the IPP/DMAPP-dependent reactions they require⁸⁸⁻⁹⁰.

The MEP pathway involves eight catalytic steps, beginning with the synthesis of 1-deoxyd-xylulose 5-phosphate (DXP) by 1-deoxy-d-xylulose-5-phosphate synthase (DXS). The next step is catalyzed by 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR), which generates MEP from DXP. MEP is then converted to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) through the action of 2-*C*-methyl-d-erythritol 4-phosphate cytidylyltransferase (MCT), 4-(cytidine 5'-diphospho)-2-*C*-methyl-d-erythritol kinase (CMK), 2-*C*-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MDS), 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS), and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR). The conversion between IPP and DMAPP proceeds through isopentenyl-diphosphate isomerase (IDI), which is a reversible reaction^{91,92}.

Although the MVA pathway is the sole source of IPP and DMAPP in many higher classes of organism, the newfound MEP pathway fulfills this role in most bacteria. Some bacteria and plants express both pathways, where they allow the organism to employ more sophisticated regulation, utilize a wider variety of precursor molecules, and synthesize isoprenoids in a broader range of environmental and chemical contexts. To date, all organisms which synthesize isoprenoids employ some combination of these two pathways. While some parasitic species contain neither complete pathway, this is only in those cases where the organisms have so thoroughly adapted to their symbiotic lifestyle that all the isoprenoid precursors they require can be obtained from their host^{93,94}.

This unusually strict separation in central metabolic pathway distribution across taxa also provides an opportunity for drug development. The absence of enzymes even distantly related to the MEP pathway constituents in animalia makes these proteins attractive targets for chemotherapeutic agents, as compounds with no off-target effects in the patient can conceivably be designed⁹⁵.



Figure 2.2 The MEP and MVA pathways. Both produce IPP and DMAPP as five-carbon building blocks for isoprenoid biosynthesis. The MEP pathway initiates with the condensation of pyruvate and glyceraldehyde 3-phosphate and an additional six steps transform DXP to IPP and DMAPP. In the MVA pathway, three molecules of acetyl- CoA condense to form HMG-CoA and an additional four steps transform HMG-CoA to IPP, which is isomerized to DMAPP. The condensation of DMAPP with one or two molecules of IPP leads to monoterpene or sesquiterpene production. From: George, Kevin W., et al. "Isoprenoid Drugs, Biofuels, and Chemicals—Artemisinin, Farnesene, and Beyond." *Biotechnology of Isoprenoids Advances in Biochemical Engineering/Biotechnology*, 2015, pp. 355–389., doi:10.1007/10_2014_288.

2.5 Isoprenoids in Metabolic engineering

Isoprenoids have many demonstrated commercial uses, including in medicine, nutrition, pigmentation, and fragrances. While plant-based sources have historically served as the principal origin of these compounds in industry, they have a variety of disadvantages for this purpose. Isoprenoids are present at only very low concentrations in most plants, therefore requiring large amounts of material and arable land to feed a process of any appreciable size. In addition, the complex chemical context that can be derived from such a feedstock makes subsequent purification steps often prohibitively expensive, as many compounds with physical properties similar to the product will inevitably be present. Chemically synthesizing these compounds instead is rarely a suitable alternative, as the relative structural complexity these compounds exhibit make doing so impractical. Because of these limitations, a significant research effort has been devoted in recent years to the development of more economical production methods through the application of metabolic engineering. Recent advances in this field, including low-cost DNA sequencing, synthesis, and an improved understanding of cellular dynamics and physiology have provided opportunities for the development of microbe-based production processes for isoprenoid manufacture⁹⁶⁻¹⁰¹.

In many examples demonstrated to date, it is the MVA pathway that has proven to be the superior platform for terpenoid production, with such systems often exhibiting better overall productivity and final product concentration. However, the MVA pathway has a more thoroughly developed body of literature to support MVA-based development, so fewer examples of processes based on the MEP pathway have so far been attempted. Nonetheless, interest in the MEP pathway remains strong, and it likely represents the future of industrial isoprenoid biosynthesis due to its several key advantages. The MEP pathway is ubiquitous throughout many

microbes that have already demonstrated a suitability for application bioprocess and amenability to genetic manipulation, including, most notably, *E coli* and the proteobacteria in general. Perhaps more importantly, the MEP pathway has a better overall stoichiometric yield, in terms of both organic carbon usage, and oxygen consumption, meaning that all other factors being equal, an MEP-based process should be able to achieve superior performance to that of an MVA-based one. It is for this reason that the engineering efforts described later in this work have been performed within the context of *E coli*, an organism possessing the MEP pathway as its sole route to isoprenoid synthesis¹⁰²⁻¹⁰⁴.

Members of the carotenoid group of compounds were among the first isoprenoids to be the subject of metabolic engineering efforts, and although they have been eclipsed in research interest by efforts towards drug production, such as the artemisinin and taxol examples described above, they nonetheless remain an important target compound. Due to their status as a natural compound with a strong red/orange pigmentation and antioxidant properties, carotenoids have found a large commercial market. Representatives from this class of compounds have been used as colorants in food and cosmetics, nutritional supplements, animal feed, nutraceuticals, and even health supplements and multivitamins. The total market for these compounds surpassed \$1 billion at the start of this decade and is expected to double by the end of the next^{105,106}.

The target compound of this work, lycopene, is one of the most valuable, with widespread use as a food coloring due to its exceptionally strong pigmentation, as a nutritional supplement due to its strong antioxidant activity, and even has shown potential as an anticancer agent. In the past two decades, lycopene has been the subject of numerous metabolic engineering efforts, and promising examples of heterologously-engineered lycopene production have been demonstrated in numerous host organisms, including *E coli*, *Y. Lipolytica*, and *S cerevisiae*. Numerous examples of engineering heterologous lycopene production in *E coli* have been demonstrated in

the literature, and impressive advancements have been made by applying a wide range of metabolic engineering techniques and strategies to this problem^{107,108}.

The enzymes encoded by dxs, idi, and mdh were found to be rate-limiting in the MEP pathway. Consequently, concurrent overexpression of these genes was found to greatly enhance lycopene production. Deletions of the gdhA, gpmAB, aceF, fdhF and gdhA showed a 40% boost in lycopene production. Although none of these genes diverts flux from the mevalonate pathway, they each provide an avenue for the oxidation of intracellular reducing equivalents. Since lycopene is a highly reduced molecule, increasing the reducing power of the cell helps its synthesis. Deleting the *E coli* zwf gene, encoding for glucose-6-phosphate 1-dehydrogenase, was shown to increase lycopene production by 130%. This enzyme catalyzes a reaction which diverts flux to the pentose phosphate pathway, and so its deletion boosts MEP pathway precursors significantly¹⁰⁹⁻¹¹¹.

Another strategy shown to yield dramatic improvements in lycopene concentration is the concurrent expression of both the native *E coli* MEP pathway, and a heterologously expressed MVA pathway from yeast. This step, and subsequently optimizing the MVA pathway resulted in 3-fold improvements in titer. The highest lycopene titer yet reported, 3.52 g/L, were achieved by combining the aforementioned steps, as well as the overexpression of several genes which boost the available ATP and NADPH pools¹¹²⁻¹¹⁵.

2.6 Dynamic Control of Metabolic Pathways

When discussing "dynamic metabolic control" as a topic in metabolic engineering, it is important to recognize that this is not necessarily a topic of research new to science, but instead represents a shift in the paradigm through which this research is pursued. A reader with even a

casual familiarity with synthetic biology and metabolic engineering will recognize that examples of dynamic regulation have existed since almost the beginning of modern molecular biology, with inducible expression under the Lac operon promoter being the best known example. However, the distinction being made in this review is a focus on research that does not simply involve what can technically be considered dynamic regulation in some way, but whose explicit aim is the improvement of process performance through engineering of the dynamic control element or system itself. Most efforts to improve strain performance, such as those discussed with respect to lycopene biosynthesis in the previous section, focus on changes to the cell that are largely static. These rely on altering the state of the cell through the inclusion of heterologous genes, deletion or downregulation of unproductive genes, and subsequently attempting to optimize the resulting strain. While these methods have resulted in enormous successes in improving fermentation yield and final titer, improvement in productivity has proven far more elusive, and it is often this quality that determines the economy of a process. Engineering through static changes alone ignores the potential and purpose of fermentative production; the process does not simply catalyze a reaction of interest, but produces catalyst as well. By relying on static changes an optimal result is not achieved, but a parsimonious one, balancing synthesis of both biomass and product, and doing each imperfectly. For these reasons, dynamic regulation has emerged as a field of increasing interest and represents a promising route by which to better balance the distribution of flux between biomass and product synthesis, and thereby overcome the limitations of the more conventional methods employed in the field¹¹⁶.

The first methods of what can be called dynamic regulation relied upon the addition of exogenous small molecules, and these methods remain central to research in the field even today. In *E coli* this has included the Lac operon with the analog of its cognate inducer, IPTG, the simple sugar arabinose with AraC, and the "Tet on" and "Tet off" systems, which induce and

repress protein expression upon introduction of tetracycline, respectively. Recent examples have demonstrated that significant improvements can be achieved by simply constructing the logic of the genetic circuit induced by a control element in a more sophisticated way, even if little change is made to the element itself. By engineering a strain of *E coli* which both upregulated expression of an isopropanol, and indirectly decrease flux through the TCA cycle, a 4-fold improvement was achieved over a strain with these properties expressed constitutively¹¹⁷⁻¹¹⁹.

Many promoter systems developed for use in yeast are dependent on substrate concentration in the media. The commonly used GAL promoter responds to the presence of galactose or raffinose, and the HXT1 promoter is induced at high concentrations of glucose. This allows a production strain to display the interesting property of controlling expression proportionally to available resources. The HXT1 promoter was used to improve fatty alcohol production by controlling the expression of free acyl-CoA and fatty acids. Lowering the strength of expression as glucose becomes exhausted improves the efficiency of the process, as resources aren't wasted on the synthesis of excess enzyme when the flux of carbon has decreased as well. The usage of small molecules as an inducer does have significant disadvantages, however. Molecules like IPTG are often prohibitively expensive for use at larger scale fermentations, and while the usage of carbohydrates as both substrate and inducer allows the engineer to design a system with what is essentially a continuously changing strength of induction, it restricts choice of media, and may be difficult to combine with other induction methods. In addition, the distribution of a small molecule inducer in a fermenter is highly dependent on its mixing properties, which at larger scales could result in a dramatic decline in the apparent responsiveness of the control element due to an uneven distribution of the inducer^{120,121}.

Another approach to dynamic expression regulation is the implementation of what is referred to as autonomous induction. This avoids many of the shortcomings of small-molecule

induction by utilizing promoters and response elements that are responsive to endogenously produced compounds, rather than those added manually. An example of this being applied in *E coli* is the optimization of MEP pathway flux. One effort to produce the antimalarial drug artemisinin involved the use of an FPP responsive promoter which governed the expression of amorphadiene, the enzyme immediately downstream of FPP. This meant that the level of amorphadiene synthase is only as much as is required to act on the available pool of FPP, and, critically, tuning the promoter can ensure that the pool of FPP is maintained at a high enough level to be used in other functions in the cell¹²².

A particularly powerful approach to applying autonomous induction takes advantage of quorum sensing. The quorum sensing system from the well-studied luminescent bacteria, *V. fishceri*, relies upon the synthesis of acyl-homoserine lactone (AHL) by the synthase LuxI. After a minimum permissive concentration of AHL has been achieved, the transcription factor LuxR binds to its cognate promoter and inducing gene expression. This provides an effective way to allow gene induction to occur only after a certain biomass concentration has been achieved, thereby allowing the fermentation to be separated into discrete biomass accumulation and product accumulation phases. Several examples of its efficacy have been shown in the literature. Another quorum sensing system, Esa QS, has been applied similarly. However, metabolic engineering efforts using Esa QS have focused on triggering induction with respect to time by making induction dependent primarily on the intracellular quorum molecule concentration, thereby partially avoiding dependence on biomass concentration^{123,124}.

Although demonstrably useful, several significant limitations to autonomous induction systems exist. These control elements are highly dependent on the metabolic context in which they are applied, so a reasonably thorough understanding of the host organism must already be known. Extensive tuning of these systems is needed as well, since many of these systems exert

what is essentially feedback control based on metabolite concentration and therefore must be made to operate within a reasonably specific dynamic range. Unlike an exogenous inducer, where expression levels can be controlled simply by adding more of the inductive signal, in autonomous induction this can only be done by adjusting factors such as promoter sequence or gene copy number, and therefore represents a fairly non-trivial engineering project in itself. In addition, this mode of induction reduces the degree of control the engineer has over induction timing and strength in an online process, which has implications on the ease of scaling and optimizing such a process.

While the examples discussed so far have been extensively applied in numerous applications, other, less orthodox methods of controlling gene expression are the subject of ongoing research or have been only recently successfully implemented. Temperature-sensitive promoters have found some use in industry, and several examples have been demonstrated. The dsra promoter encodes a small non-coding RNA involved in expression control during the *E coli* stress response. By fusing this promoter with the highly active LacUV5 promoter, a hybrid was generated demonstrating temperature-sensitivity. At 25 degrees celsius, the promoter had activity comparable to that of the wild-type LacUV5, while at 37 degrees expression levels were severely reduced. The pR/pL lambda promoter system is derived from an *E coli* phage which uses the initiation of the host heat-shock response as a trigger to initiate its lytic phase. This promoter has been shown to behave in a temperature-sensitive manner, with only low levels of production at temperatures below 37 degrees, and strong induction above this temperature. This promoter in particular has found relatively broad application for temperature-sensitive induction^{125,126}.

Recently, our own lab produced a temperature-sensitive T7-polymerase and demonstrated its use as an effective metabolic control element (Korvin and Yadav, 2018). T7 polymerase was interrupted with the temperature sensitive mutant intein described earlier, which

allowed it to function normally only at the intein's permissive temperature of splicing. Key advantages this system has over the others described here are its stringent control of expression, allowing no detectable expression to occur at 37 degrees, and its avoidance of subjecting the host to the heat-shock response required for the pR/pL promoter system to function. It is the performance of this temperature-sensitive control element that is the subject of the current work¹²⁷.

Although rarer and more recent, light-responsive control elements have been engineered as well. An engineered version of the bacterial LOV protein was expressed in a line of zebrafish embryos. This protein was designed to bind to DNA after exposure to blue light and was successfully shown to influence gene expression as an artificial transcription factor. Additionally, unlike many such artificial transcription factors, the system demonstrated both a highly linear response to the intensity of light it was exposed to, but a large dynamic range of response. Another research group demonstrated that this expression system could be used to impart extremely fine, spatially dependent control onto an engineered bacterial strain. Instead of continuous light with varying intensity, they showed that flashing the light with varying frequency afforded them greater control over gene expression^{128,129}.

3. Methods and Materials

3.1 Reagents and materials

Four strains of E. coli were assessed in this study. The strains differed only in the construction of the T7 RNA polymerase that each expresses to drive intracellular synthesis of lycopene. Cultures of all strains were propagated in Luria-Bertani (LB) medium that was supplemented with spectinomycin and ampicillin, except where otherwise noted. LB medium, spectinomycin and ampicillin were purchased from Sigma Aldrich. Molecular cloning reagents such as restriction enzymes, DNA modifying enzymes and their associated kits, and ligases were purchased from New England Biolabs. Oligonucleotides were purchased from Integrated DNA Technologies. Likewise, plasmid and PCR purification kits were purchased from Thermo Fisher Scientific. The genes of the lycopene biosynthetic pathway were cloned from the pAC-LYC plasmid¹³⁰, which was a gift from Francis Cunningham Jr. (Addgene plasmid #53270). The mutants of the VMA intein were constructed using the QuikChange Lightning site-directed mutagenesis kit purchased from Agilent.

3.2 Molecular cloning and genetic engineering

The lycopene biosynthetic pathway comprises an upstream and downstream module (Fig. 3.1). The upstream module, which is expressed natively by E. coli, comprises the nonmevalonate pathway and an additional terpene synthase encoded by the gene ispA. The non-mevalonate pathway commences with the condensation of the glycolytic intermediates, pyruvate (PYR) and
glyceraldehyde-3-phosphate (G3P) and culminates with the synthesis of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The terpene synthase subsequently synthesizes farnesyl pyrophosphate (FPP) from IPP and DMAPP. The downstream module is a heterologous pathway that is absent in E. coli. The module comprises three genes, crtE, crtB and crtI that successively transform FPP to GPP, phytoene (PHY) and lycopene (LYC), respectively. The genes of the downstream module were cloned from the pAC-LYC plasmid using PCR and inserted downstream of a T7 promoter in a pET plasmid that has a pBR322 origin of replication and an ampicillin selection marker. Since the T7 operator region in was not included in construct, IPTG is not required for induction of the lycopene pathway. Instead, only formation of active T7 polymerase is sufficient for this induction. The resulting plasmid is labeled as pLYC. Likewise, the genes encoding the VMA intein and the T7 polymerase were directly cloned from S. cerevisiae and T7 phage particles also using PCR. The intein and polymerase comprise 454 and 883 amino acid residues, respectively. The genetic sequence of the intein was subsequently edited using site-directed mutagenesis to construct the temperature-sensitive (N454Q) and non-excisable or dead (L212P) mutants (Fig. 2B). A previous study had determined that while the wild-type VMA intein excises itself from the host protein at comparable rates at any temperature between 18-37 °C, the temperature-sensitive intein can only excise itself at temperatures at or near 18 °C. Nevertheless, the excision rate is highest at 18 °C²³. The genetic sequences of each intein variant were then recombined into the sequence of the polymerase using Golden Gate assembly of PCR products bearing SapI restriction sites. All three intein variants were inserted between A491 and C492 of the polymerase to yield a 1337-residue protein. The complete genetic sequences of the unmodified and modified polymerases were later introduced downstream of a lac promoter into a pZS4 plasmid bearing a pSC101 origin of replication and a spectinomycin selection marker. The lac promoter is also induced with IPTG.

The plasmid that harbors the unmodified T7 RNA polymerase is labelled as pRNAP, whereas the plasmids expressing the three modified polymerases are labelled as pRNAP-WT (wild-type intein), pRNAP-TS (temperature-sensitive intein) and pRNAP-Dead (non-excisable intein). The gene encoding the enhanced green fluorescent protein (eGFP) was also inserted analogously into a separate pET plasmid having the same pBR322 origin of replication and ampicillin selection marker described earlier. The resulting plasmid is designated as pGFP and was employed to confirm activities of the intein-modified polymerases in cultures of strains that do not harbor the lycopene biosynthesis pathway. Eight investigational strains were generated in this study by cotransforming E. coli with any one of pRNAP or pRNAP-WT or pRNAP-TS or pRNAP-Dead and either the pGFP or pLYC plasmids. An additional strain was co-transformed with pRNAP and an empty pGFP plasmid for use as a normalization standard for growth and fluorescence measurements. Culturing conditions All E. coli cultures were propagated in 100 mL of LB medium supplemented with glucose, spectinomycin, ampicillin and IPTG at concentrations of 1 mg mL-1, 50 µg mL-1, 100 µg mL-1 and 0.1 mM, respectively, unless otherwise noted. Each of the cultures had a starting optical density (measured at 600 nm on a Perkin Elmer Envision 2101 multi-label microplate reader) of 0.05 and was agitated at 200 rpm.



Figure 3.1 The metabolic pathway to lycopene biosynthesis. The enzymes and compounds presented in gray represent those involved in the native MEP pathway, while those in orange are a part of the heterologously expressed lycopene biosynthesis gene cassette, whose expression is controlled in this work by the experimental T7 polymerase constructs. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.

3.3 Growth and activity assays

Optical densities of the cultures were measured at 600 nm every 30 minutes over six hours. Cultures expressing the pGFP plasmid were excited at 480 nm and the emitted fluorescence was detected at 530 nm with the gain of the microplate reader set to 10. Fluorescence was also recorded every 30 minutes over six hours. The optical density and fluorescence measurements for the cultures at each time point were normalized by calculating the ratio to the readout for cultures of a strain co-transformed with pRNAP and an empty pGFP plasmid. Fieller's theorem¹³¹ was employed to estimate the 95% confidence interval for the ratio of the mean value at each time point. Briefly, the standard errors in the means of the measurements (SEM) for two populations are:

(1)
$$\operatorname{SEM}_{A} = \frac{\sigma_{A}}{\sqrt{n_{A}}}$$

(2)
$$\operatorname{SEM}_{\mathrm{B}} = \frac{\sigma_{\mathrm{B}}}{\sqrt{n_{\mathrm{B}}}}$$

Here, μA and μB are the mean values for the two populations, whereas σA and σB are their corresponding standard deviations. The terms nA and nB refer to the number of independent observations. Consequently, the 95% confidence interval for ratio of the mean values is estimated as:

(3)
$$\operatorname{SEM}_{Q} = \frac{\mu_{A}}{\mu_{B}} \sqrt{\left(\frac{\operatorname{SEM}_{A}}{\mu_{A}}\right)^{2} + \left(\frac{\operatorname{SEM}_{B}}{\mu_{B}}\right)^{2}}$$

(4)
$$\frac{\mu_{\rm A}}{\mu_{\rm B}} \pm 1.96 \cdot \rm{SEM}_{\rm Q}$$

Owing to difficulties associated with reliably quantifying lycopene directly in the culture medium, 10 mL of the lycopene cultures were initially centrifuged and resulting pellets were resuspended in 2 mL of deionized water. This solution was then vigorously contacted with 4 mL of acetone in a vortex mixer for 20 minutes. Finally, 3.8 mL of the organic fraction was

withdrawn, dried in air and resuspended in 100 μ L of ethyl acetate for quantification by absorbance at 481 nm. Unless otherwise indicated, cultures were replicated 15 times to estimate the mean of the optical density, fluorescence and lycopene readout at each time point. All glucose measurements were done after ultrafiltration of culture supernatant from each timepoint noted, and performed by HPLC on an Aminex HPX-87C Carbohydrate Analysis Column, as described in Parpinello and Versari (2000)¹³². All the culturing experiments described in section 4.5 were done using M9 minimal media and supplemented with 10 g/L of glucose as the sole carbon source, and 20 mM of ammonium chloride as the sole nitrogen source.

4. Results and Discussion

4.1 Molecular Biology and DNA Assembly

To construct the 4 modified T7 RNA plasmids, DNA fragments encoding the VMA intein were obtained from *Saccharomyces cerevisiae* cells by PCR. This step was used to both simultaneously obtain adequate amounts of DNA for assembly downstream, and to introduce point mutations into the intein DNA, where necessary. To obtain the TS intein, this sequence was amplified in two separate amplicons so that the mutation could be introduced as 5' noncomplementary primer overhang in a standard PCR. Inverse PCR was performed on a plasmid containing a gene encoding T7 polymerase (pRNAP), with primers chosen to "open" the RNA polymerase at the point required to insert the VMA intein DNA. This created a linear fragment with sites amenable to the modified Gibson's assembly described in methods. Each of these amplifications, and the success of the subsequent assemblies were verified by agarose gel electrophoresis (Fig. 4.1).

To determine if the assembled constructs are expressed correctly, and that intein splicing occurs as designed, cell lysates expressing each of the 3 intein-containing constructs were run on an SDS-PAGE gel for analysis (Fig 4.2). The cultures were grown at 37 °C, but the lysates and gels were prepared and run at room temperature, approximately 23 °C, with the exception of lane 3, which contains the temperature-sensitive intein, and so an attempt was made to maintain it at 37 °C so as to demonstrate its inability to fold.

Examination of the pRNAP-wt lysate showed two discrete bands, one for the reconstituted T7 RNA polymerase and one for the spliced intein, at around the expected mass

regions of approximately 99kDa¹³³ and 50kDa¹³⁴, respectively. This indicates that the unmodified intein construct behaves largely as expected, excising itself from its host RNA polymerase. In contrast, the pRNAP-dead intein lysate exhibited only a single relatively strong band at around the mass region of approximately 150 kDa. This corresponds roughly to the sum of the molecular mass of T7 polymerase and the VMA intein, indicating no splicing could occur. The results from the pRNAP-TS lysate are less clear, however. The two bands indicating splicing are clearly present, however, a 3rd band seems to exist at the mass corresponding to the dead intein. The high background associated with the unpurified lysate makes it difficult to determine with certainty whether the unspliced band is present at all. However, although this TS intein mutant has been shown to have negligible splicing at 37 °C, it has also been shown to splice at some temperatures above its permissive temperature of 18 °C, though less quickly⁷⁰. Although maintaining the temperature at 37 degrees was attempted for the lane 3 sample, reliably maintaining this temperature at all points during the assay was impractical, as many of the techniques involved require conditions below 37 °C, if only briefly. In addition, relatively fast splicing has been demonstrated for the mutated intein^{70,133}, and therefore these multiple bands could just represent populations of both spliced and unspliced proteins from a reaction that was allowed to partially complete at those points when sub-37°C temperatures were required.



Figure 4.1 Agarose gel electrophoresis after PCR amplification of DNA fragments used in this work. DNA amplicons are from left to right, as indicated in the figure: unaltered wild-type VMA Sce intein, the non-functional dead intein, the temperature-sensitive version of the intein amplified in two fragments for assembly, TSI-1 and TSI-2, the full length T7 RNA polymerase, and T7 polymerase amplified in two fragments for assembly, T7 fragment and T7 fragment 2.



Figure 4.2 SDS-PAGE gel exhibiting lysates from 3 expression strains. Lane 1 contains pRNAPwt strain lysate, lane 2 pRNAP-Dead lysate, lane 3 contains pRNAP-TS lysate. The 3 bands of interest, as indicated, are the T7 polymerase enzyme interrupted with intein at 150 kDa, the uninterrupted T7 polymerase at 99 kDa, and the spliced intein at 50 kDa. The image is presented in grayscale for clarity.

4.2 Temperature-Sensitive Intein Validation

To confirm whether the four experimental plasmid constructs pRNAP, wild-type intein (pRNAP-WT), temperature-sensitive intein (pRNAP-TS), and dead intein (pRNAP-Dead), were working as designed, they were each coexpressed with a second plasmid, pGFP, expressing eGFP under the control of a T7 promoter. Since the intein construct containing the temperature sensitive intein, pRNAP-TS, will only correctly excise itself from within RNA polymerase at the lower permissive temperature of 18 °C, expression of eGFP should only be able to occur at this condition as well. Competent *E coli* cells were cotransformed with each of the experimental plasmids and pGFP, and subsequently grown overnight on LB agar at the indicated temperature (Fig 4.3). Critically, this experiment provides clear evidence that the mutation introduced into pRNAP-TS allows expression to occur at the permissive temperature of 18 °C, and no detectable expression is allowed to occur at the restrictive temperature of 37 °C as comparison with the positive and negative controls of pRNAP and pRNAP-Dead indicate.

While this experiment does provide only indirect evidence that the intein has folded and excised correctly, in many ways this information is ultimately more useful than direct evidence of the intein's performance. In order to act as a useful component within a larger metabolic control network, the performance of the temperature-sensitive construct within the context of a typical *in vivo* expression system must be investigated. This experiment not only provides some insight into that performance, but the fact that no eGFP was detected in either positive control also proves that the stringency of expression control provided by the temperature-sensitive intein is maintained over the entire period of culture growth.

To provide further insight into the performance of the temperature-sensitive intein construct, the growth and fluorescence of the strains containing each of the four coexpressed

plasmids were measured over time. First, the growth, measured as absorbance at 600nm, was measured concurrently with fluorescence, excited at 480nm and detected at 520nm. These cultures were maintained at a temperature of 37 degrees, and measurements were conducted over a period of 18 hours (fig 4.4). The normalized fluorescence at this temperature confirms that the unmodified polymerase and its variant bearing the wild-type intein are significantly more active than the polymerases bearing the temperature-sensitive and non-excisable inteins. An interesting result seen here is that the expression profile of the pRNAP and pRNAP-Dead strains is almost indistinguishable. This suggests that the increased metabolic burden placed on the cell by the expression of the larger, intein-containing version of T7 RNA polymerase is only a trivial amount larger than that of the wild type enzyme, a result important for its implications on the suitability of the intein as a component of a metabolic control system. Since as many as 20-100 copies of the pGFP plasmid are maintained within the cell and the transcriptional activity of a functional T7 polymerase is quite high¹³⁵, the optical densities of the former two strains are also much lower than the strains that express inactive polymerases owing to an increase in metabolic stress that is incurred by the expression of eGFP. The plasmid containing the T7 RNAP variants is maintained at a much lower copy number, approximately <10, and due to the much higher activity of the T7 RNAP relative to the native polymerase a non-trivial fraction of the cell's dry weight can probably be accounted for by eGFP at the end of the culture period.

It has previously been shown in experiments using similar conditions that protein under the expression control of T7 RNAP can account for as much as 40% of the cell's mass¹⁸, even where the polymerase is present at levels of less than 1000 enzymes per cell, making the substantial difference in growth over the measured period an unsurprising result, despite the relative simplicity and small mass of eGFP. Unlike the pRNAP and pRNAP-WT strains, pRNAP-Dead and pRNAP-TS showed a largely indistinguishable phenotype, strongly suggesting

that if any excision of the temperature-sensitive intein is occurring above the restrictive temperature, it's below the threshold needed for any detectable level of eGFP to be seen.

Using otherwise identical conditions, the culturing experiment with all four strains was performed again at 18 °C. In contrast to the previous culturing experiment, fluorescence emitted at this temperature by the strain expressing the temperature-sensitive intein is nearly identical to that emitted by the strains expressing the unmodified polymerase and the polymerase interrupted by the wild-type intein (Fig. 4.5). Additionally, while growth of the strains expressing eGFP was lower at both 37 °C and 18 °C, the difference was much wider at the lower temperature. Since the cells have had a severe reduction in growth at 18 °C, they are able to allocate nearly all cellular resources that are available for growth to expression of eGFP, which greatly lowers their growth compared to the strain that expresses the polymerase bearing the dead intein or the one that does not express eGFP at all. In addition, it has been previously shown that cultures expressing heterologous enzymes have a more strongly depressed growth rate at lower temperatures, and that inducing expression during the lag or early exponential phase of growth, as was the case here, has a much larger effect on growth rate than induction during the late exponential phase¹³⁶.



Figure 4.3 1.5% LB agar plates with each of the experimental *E coli strains*, grown overnight at 18 and 37 degrees celsius as indicated. The plates denoted as temperature-sensitive contain strain expressing the temperature-sensitive inteins, positive control 1 and 2 are expressing the uninterrupted T7 polymerases and wild-type inteins, respectively, and the negative control contains the T7 polymerase interrupted with a dead intein.



Figure 4.4 Normalized activity of experimental strains at 37 degrees celsius. The presented data are OD600 and fluorescence emitted by one of each of the four strains, pRNAP, pRNAP-WT, pRNAP-TS, pRNAP-Dead, coexpressed with pGFP. To obtain a more valuable representation of the strains' performance, and to reduce the obfuscating effect of the low-level fluorescence associated with biomass, the data presented are not the absolute values measured, but are normalized relative to measurements obtained from a 5th strain expressing none of the 4 plasmids. The shaded regions around each line of data represents the associated 95% confidence interval. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.



Figure 4.5 Normalized activity of experimental strains at 18 degrees celsius. The presented data are OD600 and fluorescence emitted by one of each of the four strains, pRNAP, pRNAP-WT, pRNAP-TS, pRNAP-Dead, coexpressed with pGFP. To obtain a more valuable representation of the strains' performance, and to reduce the obfuscating effect of the low-level fluorescence associated with biomass, the data presented are not the absolute values measured, but are normalized relative to measurements obtained from a 5th strain expressing none of the 4 plasmids. The shaded regions around each line of data represents the associated 95% confidence interval. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.

4.3 Productivity Improvement of Lycopene

The expression of eGFP under the control of the temperature-sensitive intein represents a valuable case study by which to investigate the activity of the system as a component of an *in* vivo expression system, and how it performs within the context of a culture over a relatively long growth period. However, to truly gauge the utility of the intein as a metabolic control element, it becomes necessary to apply the intein to the synthesis not only of individual proteins, but to more complex gene cassettes governing the generation of secondary metabolites, because such a context provides more thorough evidence of its performance in an environment of increased complexity, where unforeseen interactions between components of the system can impart potentially problematic emergent properties onto the system. Perhaps even more significantly, the increased metabolic burden imposed on the host cell by the greater number of genes and their catalytic activity means that more strictly delineating a growth phase from a product synthesis phase, which is the ability conferred onto the system by the temperature-sensitive intein, has a far greater impact on the performance of the system overall, and therefore represents a use case capable of emphasizing the potential of such a control element. The terpenoid lycopene was chosen as the secondary metabolite of interest for this phase of experimentation, as there are a number of factors that make it particularly compelling for this purpose. As a terpenoid, lycopene represents a class of molecules of great research interest and significant economic potential, and therefore any advancement in lycopene metabolic engineering could likely be applied to an already very active area of research^{107,111}. In addition, because lycopene is itself a compound of some commercial significance, numerous examples of the expression of lycopene synthesis genes in *E coli* exist in the literature, and so provides a well-studied system to investigate as a part of this work. Indeed, the particular cassette of lycopene synthesis genes used here has been used in

numerous studies already, and as such has been thoroughly characterized¹³⁰, providing a great deal of valuable information as how it should be expected to perform, and serving as a contrast to how the intein-based control element used here impacts lycopene synthesis. Finally, lycopene has a distinct red color, imparting a pigmentation phenotype onto cells expressing it. Lycopene has three relatively discrete and strong absorbance peaks, at 444nm, 471nm, and 503nm, and as such lycopene's pigment properties can be used to provide a method of reliably estimating its concentration in a solution without resorting to more involved and time-consuming methods like HPLC or GCMS¹³⁷.

To investigate the temperature-sensitive intein's performance while controlling production of a secondary metabolite, cultures of E. coli strains that co-express any one of the 4 T7 RNA polymerase plasmids (pRNAP, pRNAP-WT, pRNAP-TS or pRNAP-Dead) and the lycopene gene cassette-containing plasmid pLYC were evaluated. The initial optical density of each culture following inoculation was 0.05, a concentration high enough that the growth of the culture should approach stationary phase over the timeframe chosen for the experiment, but not so high that the different phases of growth relevant to the expression of heterologous genes in Ecoli aren't clearly seen in the data. The cultures were propagated at 37 °C until their optical densities increased to 0.5. Although the cells should, in theory, not transcribe the coding sequence of either of the two plasmids they contain, the T7 promoters under the control of the lac operator exhibit a relatively high basal expression level¹³⁸. This is thought to be a consequence of both the inability of the LacI repressor to perfectly restrict transcription at Lac controlled promoters, and the very high activity of T7 RNA polymerase and affinity for its promoter. As a result, even if even a relatively low concentration of the enzyme is present in the cell it can result in an expression phenotype defined not only by detectable levels of product, but that in some cases approaches or matches levels after induction. Therefore, resources that would otherwise be

exclusively allocated towards growth during this early stage of the fermentation are actually divided between growth, expression of the T7 polymerase, expression of the lycopene pathway, and synthesis of lycopene, if the cell expresses an active variant of the T7 polymerase. Expression of the lycopene pathway has, in particular, an especially detrimental effect on the growth of the cell, as they not only divert carbon and nitrogen that could be used for biomass towards protein synthesis but divert flux from the MEP pathway that would otherwise go towards the synthesis of metabolites which perform vital functions to the health of the cell. This toxic effect of heterologous expression on MEP-pathway related functions has been shown to be especially prominent when the heterologous pathway is the lycopene gene cassette¹³⁹. Biomass accumulation in cultures expressing pRNAP and pRNAP-WT is markedly lower than cultures expressing Q4 pRNAP-TS and pRNAP-Dead (Fig. 4.6).

IPTG was added to the medium and the fermentation temperature was lowered to 18 °C once the optical density of a culture reached 0.5, formally initiating lycopene production in all cultures with the exception of cells expressing pRNAP-Dead. It was observed that the final concentration of lycopene increased with respect to the concentration of IPTG used as inducer until about 0.05 mM IPTG, after which little improvement or even a detrimental effect was seen (Fig 4.7).

Therefore, the final concentration of IPTG was adjusted to 0.05 mM in order to minimize the metabolic stress experienced by the host resulting from unnecessarily strong induction. The strain expressing pRNAP-TS is approximately 15% more productive than the strains expressing pRNAP and pRNAP-WT, in terms of final concentration attained over the period, and that it also completely suppresses lycopene production prior to induction (Fig. 4.8).

Additionally, although the optical densities of all cultures with the exception of the strain expressing pRNAP-Dead eventually converge, lycopene production by the strain expressing

pRNAP-TS is consistently higher. This observation confirms that the pRNAP-TS strain separates biomass accumulation and product formation in the intended manner, though imperfectly since the temperature drops from 37 °C to 18 °C gradually. The imperfect demarcation of growth resulting from the limitations of using temperature as an induction signal likely contributes to the appearance of Luedeking–Piret¹⁴⁰ as opposed to growth-independent lycopene production kinetics. It is likely, then, that a T7 RNA polymerase that is interrupted with a chemo- or photoexcisable intein might separate growth and product formation more strictly. Finally, bacterial terpenoid synthesis comprises an upstream and downstream module, and the nonmevalonate, or MEP pathway, which makes up most of the upstream module, is common to all terpenoid biosynthetic pathways. The enzymes on the pLYC plasmid, encoded by the genes dxs, ispD, ispF and idi have previously been shown to catalyze the rate-limiting steps in the nonmevalonate pathway¹⁰⁹. These enzymes are poorly soluble and typically form inclusion bodies within the host cell, particularly when they are overexpressed or are integrated downstream with overexpressed heterologous pathways. Kim *et al.* previously determined that lowering the fermentation temperature to 25 °C or lower facilitated improved solubilization of the rate-limiting enzymes, which improved productivity by nearly 20%. If it is assumed that this same result holds true in the context in which the lycopene pathway is expressed in this work, then the combination of a temperature downshift with the strict demarcation of growth and product formation seen in this work could result in as much as a 38% improvement over the culture expressed at 37 °C without the aid of the intein control element.



Figure 4.6 Growth comparison of the 4 experimental strains while coexpressing a lycopene expression gene cassette. The data are measured OD600 over time by one of each of the four strains, pRNAP, pRNAP-WT, pRNAP-TS, pRNAP-Dead, coexpressed with pLYC. A temperature-shift was performed on each culture once an OD of 0.5 was reached, roughly 250 minutes for pRNAP-TS and pRNAP-Dead, and 470 minutes for pRNAP and pRNAP-WT. The shaded region represents the 95% confidence interval of the associated data. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.



Figure 4.7 Relative impact of IPTG concentration on product concentration. The pRNAP strain coexpressing pLYC was cultured overnight at the indicated concentrations of inducer. Error bars represent a 95% confidence interval and are based on 3 replicates.



Figure 4.8 Product synthesis comparison of the 4 experimental strains while coexpressing a lycopene expression gene cassette. The data are the measured absorbance at 481 nm from samples taken over time by one of each of the four strains, pRNAP, pRNAP-WT, pRNAP-TS, pRNAP-Dead, coexpressed with pLYC. A temperature-shift was performed on each culture once an OD of 0.5 was reached, roughly 250 minutes for pRNAP-TS and pRNAP-Dead, and 470 minutes for pRNAP and pRNAP-WT. The shaded region represents the 95% confidence interval of the associated data. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.

4.4 Predictive Model Development and Analysis

The ability to strictly separate a biomass accumulation phase from product synthesis has been highly sought after in the field of metabolic engineering¹⁴³. As demonstrated in the previous sections, being able to do so results in a notable increase to the productivity of the process, even when no other change to the metabolism and physiology of the host cell is made. A variety of attempts to solve this problem have been attempted, a few noteworthy examples of which are described in the literature review section of this work. These attempts typically suffer from three main weaknesses: the imperfect demarcation of growth and product accumulation, the method of induction requires prohibitively expensive chemicals for induction, as with small molecule inducer-based control, or the inability to respond to external stimulus, thereby limiting control options for the process engineer, as with autoinduction. Examples avoiding both of these issues, such as the temperature-sensitive intein described in this work, are relatively rare, but their obvious advantages mean that they remain an attractive subject for further study. It is therefore worth considering how hypothetical examples of this type of dynamic external control system would likely affect a bioprocess.

To theoretically demonstrate the utility of the temperature-sensitive intein, or other similar metabolic control element, and how it would likely perform within the context of a bioprocess, a model based on the Monod¹⁴⁴ and Luedeking-Piret¹⁴⁰ equations describing growth, substrate utilization, and product synthesis was developed. It was then used to predict the likely sensitivity of the overall bioprocess to perturbations in a number of relevant parameters. These parameters are the length of the pre-induction biomass accumulation phase (Fig 4.9), yield of biomass and product on substrate (Fig 4.10), and culture growth rate and specific rate of product formation (Fig. 4.11). While the first variable can be controlled as a part of any system of this

type, the latter two are dependent on the particular host organism used, the product synthesis pathway of interest, and the kinetics of the particular induction stimulus and system used.

The equations (5) and (6) shown below describe the growth of the strain that co-expresses pRNAP and pLYC. More generally, these equations describe the growth and substrate consumption of some strain, denoted here by the subscript 1, heterologously engineered to produce some metabolite of interest, but that does not contain an externally-controlled dynamic metabolic control element of the type described in this work.

(5)
$$\frac{\mathrm{d}X_1}{\mathrm{d}t} = \frac{\mu_{\max} \cdot S_1 \cdot X_1}{K_s + S_1}$$

(6)
$$\frac{\mathrm{d}S_1}{\mathrm{d}t} = -\frac{Y_{\mathrm{S}/\mathrm{X}_1} \,\mu_{\mathrm{max}} \,S_1 \,X_1}{K_{\mathrm{S}} + S_1}$$

 (X_1) and (S_1) are defined here as the rate of biomass accumulation and substrate consumption, respectively. Specifically, they describe the activity of strain 1, the uncontrolled strain. In the equations above, μ_{max} , K_s and Y_{sx^1} represent the maximum specific growth rate, the concentration of substrate at which the specific growth rate is half of its maximum value, and the ratio of the mass of substrate consumed to the mass of biomass produced during the fermentation, respectively. In theory, no product should be synthesized during this phase. Following induction during the second half of the exponential phase of growth, and more specifically at an OD of 0.5 for most of the experimental examples discussed here, the cultures commence synthesis of the product. Since the formation of product by the cultures is directly correlated with the intracellular concentration of the heterologously expressed enzymes, it is associated with growth, particularly when the cultures are not limited by the availability of substrate¹⁴⁵. The degree of growth association the synthesis of a particular product has is a consequence of a variety of different factors. What role the product has in the physiology of the host, such as ethanol or lactic acid which are produced as a direct result of the cell's energy metabolism, can often be a driving factor in this association¹⁴⁶. In addition, there are pronounced differences in cellular physiology during rapid growth that lead to this phenomenon of growth association such as the far higher level of ribosomes per cell seen during the exponential phase, which lends itself to more rapid protein synthesis¹⁴⁷. Consequently, the rate of substrate consumption and product formation are represented as:

(7)
$$\frac{\mathrm{d}S_{1}}{\mathrm{d}t} = -\frac{Y_{\mathrm{S}/\mathrm{X}_{1}} \cdot \mu_{\mathrm{max}} \cdot S_{1} \cdot X_{1}}{K_{\mathrm{S}} + S_{1}} - \frac{Y_{\mathrm{S}/\mathrm{P}_{1}} \cdot Y_{\mathrm{P}/\mathrm{X}_{1}} \cdot \mu_{\mathrm{max}} \cdot S_{1} \cdot X_{1}}{K_{\mathrm{S}} + S_{1}}$$

(8)
$$\frac{\mathrm{d}P_1}{\mathrm{d}t} = \frac{Y_{\mathrm{P}/\mathrm{X}_1} \cdot \mu_{\mathrm{max}} \cdot S_1 \cdot X_1}{K_{\mathrm{m}} + S_1}$$

 Y_{sp^1} represents the ratio of the mass of substrate consumed to the mass of product that is synthesized, whereas Y_{px^1} represents the ratio of the mass of product synthesized to the mass of biomass produced. In contrast, the second, experimental strain coexpressing pRNAP-TS and pLYC is defined in the equations below. Just as with the above equations, (X₂) and (S₂) represent biomass accumulation and substrate consumption, but now represent the activity of the second, dynamically controlled strain, as denoted by the subscript 2. Otherwise, these equations are identical to (5) and (6).

(9)
$$\frac{\mathrm{d}X_2}{\mathrm{d}t} = \frac{\mu_{\max} \cdot S_2 \cdot X_2}{K_{\mathrm{S}} + S_2}$$

(10)
$$\frac{\mathrm{d}S_2}{\mathrm{d}t} = -\frac{Y_{\mathrm{S/X_2}} \cdot \mu_{\mathrm{max}} \cdot S_2 \cdot X_2}{K_{\mathrm{S}} + S_2}$$

Since the metabolic burden that is incurred by the two strains in order to maintain the coexpressed plasmids is only trivially different, µmax can be considered equal for both strains. Similarly, X₁ and X₂, and S₁ and S₂ are also considered identical prior to induction for the purposes of this analysis. As designed, the strains express their respective form of polymerase after chemical induction, and the temperature-sensitive polymerase of strain 2 is subsequently activated when the temperature of the cultures is lowered from 37 °C to 18 °C. This shift initiates product formation, which can be considered as largely independent of growth at 18 °C, since E. coli enters an approximation of stationary phase under these conditions^{148,149}. This new relationship between growth and product formation is therefore assumed to follow Luedeking– Piret kinetics. The rates of biomass accumulation, substrate consumption and product formation by cultures of the second strain are:

(11)
$$\frac{\mathrm{d}X_2}{\mathrm{d}t} = m$$

(12)
$$\frac{\mathrm{d}S_2}{\mathrm{d}t} = -Y_{\mathrm{S}/\mathrm{X}_2} \cdot m - Y_{\mathrm{S}/\mathrm{P}_2} \cdot \alpha \cdot m - Y_{\mathrm{S}/\mathrm{P}_2} \cdot \beta \cdot X_2$$

(13)
$$\frac{\mathrm{d}P_2}{\mathrm{d}t} = \mathbf{\alpha} \cdot \mathbf{m} + \boldsymbol{\beta} \cdot \boldsymbol{X}_2$$

An important aspect of equations 12 and 13 is the replacement of the yield coefficient Y_{px} with the growth-associated product formation coefficient α . This is to avoid confusion over the identity of these terms, where Y_{px} accounts for all product formation with respect to biomass, whereas α accounts only for that product associated with biomass formation. If, as in equation 8, the growth associated term and specific rate of formation term are not separated, a Y_{px2} term could still be defined. In the equations above, biomass is assumed to accumulate at rate m, as defined by equation 11, and β represents the specific rate of formation of product that is independent of growth rate. Several dimensionless variables are used in figures 4.9, 4.10, 4.11, and 4.12 (table 4.1). Dimensionless time, θ is defined as the time elapsed over the time of induction. Also, the ratios of the biomass and product concentrations of strain 2 to strain 1 are defined as χ and π , respectively, and assume that cultures of both strains contained equal amounts of starting substrate. Likewise, the ratio of Y_{sx^2} to Y_{sx^1} is ψ_s , and the ratio of Y_{px^2} to Y_{px^1} is ψ_p . As would be expected, for any value of Y_{sx} and Y_{px} , the second strain, whose heterologous pathway

expression is under the control of a hypothetical metabolic control element, has less biomass accumulated after induction occurs than the uncontrolled strain.

This trend is seen throughout figures 4.9, 4.10 and 4.11, as one of the key properties desired in any such metabolic control system is the strict demarcation of growth and product formation, with defined and discrete biomass and product accumulation phases. Similarly, metabolic engineering of the controlled strain, which typically improves the yield of product (Y_{DX}) and minimizes the yield of biomass (Y_{SX}) , further enhances its productivity over that of the first strain (Fig. 4.9)¹⁵¹. A key parameter that can vary amongst different metabolic control strategies is how abruptly the system will respond to its triggering signal. That is, how quickly after the external stimulus required to activate the metabolic control element is provided, in the case of the intein system, temperature, the desired change in the system's activity will be seen. In quantitative terms, this faster system response time corresponds to a lowering of the Luedeking-Piret apparent growth rate, m, as biomass accumulation is more strictly reduced after induction, and to an increase in relative product formation β . While this relationship is not immediately intuitive, it results from the fact that in a system with slower response kinetics, the distinction between the growth phase and product is less clear, and therefore leads to an apparent decline in β for the phase as a whole. The improved performance represented by the increase in β and decrease in m also results in a significant increase in the relative productivity of the controlled strain as well, as should be expected (Fig. 4.11).

Lastly, delaying the induction of the heterologous pathway also improves the relative productivity of the controlled strain, although, paradoxically, overall relative biomass accumulation for the engineered strain is also higher compared to cases where induction is much earlier (Fig. 4.9). The impact of this particular parameter is likely highly dependent on the

context in which it is used. Fortunately, the the veracity of this model as it applies to the temperature-sensitive intein can at least be verified experimentally.

To verify experimentally the parameters adjusted for the first two models, yield, growth rate, and specific rate of product formation, would require a substantial amount of additional metabolic engineering to be applied to the host organism, the heterologously expressed pathway, or the use of a completely different metabolic control method that would allow substantive changes to be made to the bioprocess conditions, and is therefore beyond the scope of this work. However, the third model, time of induction, is largely independent of host, method, or product, and therefore is possible to verify in this system simply by inducing with IPTG and a temperature shift at varying times. While the results largely obeyed the overall trend established in figure 4.9, there are obvious discrepancies between the experimental data and the model, which serve to both emphasize the limitations of the model as it is written, thereby informing efforts to improve upon it, and to provide valuable insight into the performance of the temperature-sensitive intein itself (Fig 4.12).

It is immediately clear after consideration of figure 4.12 that the model fails to adequately consider the impact that "leaky" expression¹⁵¹, one of the key advantages the temperature-sensitive intein confers, has upon this system. Rather than remaining equal before induction, the premature expression of the lycopene gene cassette in the uncontrolled strain results in a dramatic reduction in growth of the uncontrolled strain even before induction. Indeed, before induction strain 2 has a measured OD over twice that of strain 1. In addition, the model underestimates the negative impact on growth rate resulting from induction. However, in this particular example, this effect is likely amplified due to the temperature shift involved, since it has been demonstrated that the impact of heterologous protein expression on host growth rate is increased by lower temperature and increased further by induction in an early growth phase¹³⁶.

The model also underestimates the improvement in product formation rate resulting from induction at a later time point. As seen in the next section, the culture has a stronger dependence on specific product formation rate β , than growth-associated product formation α , making this result less surprising. Whether the relative importance of β over α is also exaggerated by the temperature shift is not directly explored in this work, but because of the significant decline in growth rate seen at the lower temperature it is nonetheless likely.



Figure 4.9 Dimensionless biomass and product formation ratio of a hypothetical dynamically controlled strain vs a conventional expression strain. The dimensionless time points 0.5θ and 2θ refer to a time of induction occurring at half the time and twice the time it takes for strain 2 to reach its normal induction OD of 0.5. Evidently, delaying induction improves the performance of a system possessing a metabolic control mechanism. In a real culture however, it can be predicted that delaying induction by much longer would have a strongly detrimental impact on performance, either due to reaching a prohibitive cell density or substrate depletion. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.



Figure 4.10 Dimensionless biomass and product formation ratio of a hypothetical dynamically controlled strain vs a conventional expression strain. The performance of a strain 1 and 2 with several different biomass and product yields, which is a desired a typical outcome of metabolic engineering, is investigated. The biomass ratio of the two strains remains unchanged, while the improvement in productivity imparted by the control system in strain 2 is improved, weakly with respect to biomass yield, strongly with respect to product yield. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.



Figure 4.11 Dimensionless biomass and product formation ratio of a hypothetical dynamically controlled strain vs a conventional expression strain. Here, the impact of altering the apparent growth rate m, and specific product formation rate β is investigated. These parameters can be altered in some cases by pathway engineering, as well as bioprocess conditions, and the specific metabolic control strategy applied. The TS intein, for example, would lower m by virtue of lowering temperature as a part of its use. Unsurprisingly, maximizing both parameters serves to complement a metabolic control strategy, as such a system would allow the process engineer to raise m initially to accumulate biomass, and subsequently lower m and raise β to encourage maximum product formation. From: Korvin, Daniel, and Vikramaditya G. Yadav. "A Molecular Switch That Enhances Productivity of Bioprocesses for Heterologous Metabolite Production." *Molecular Systems Design & Engineering*, vol. 3, no. 3, 2018, pp. 550–559., doi:10.1039/c8me00013a.



Figure 4.12 Experimental validation of the impact of induction time on the performance of a strain with product synthesis controlled by a dynamic control element. Although the premature formation of product by strain 1 has an unexpectedly large impact on biomass accumulation, delaying induction results in improved productivity, as predicted.

X1, X2	Biomass of strain 1 or 2, measured in OD600
S1, S2	Substrate (glucose) concentration in culture of strain 1 or 2, measured in g/L
P ₁ , P ₂	Product (lycopene) concentration in culture of strain 1 or 2, measured in absorbance at 481 nm.
Ks	Monod substrate affinity constant, measured in g/L of substrate
μ_{max}	Maximum growth rate of culture, measured in h ⁻¹
Y_{sx^1}, Y_{sx^2}	Substrate yield coefficient of strain 1 or 2 on substrate, measured as $(g/L)/(OD600)$.
Y_{px1}, Y_{px2}	Product yield coefficient of strain 1 or 2 with respect to biomass, measured as (absorbance at 481 nm)/(OD600).
Y_{sp1}, Y_{sp2}	Substrate yield coefficient of strain 1 or 2 with respect to product, measured as $(g/L)/(absorbance at 481 \text{ nm})$.
α	Growth-associated product formation coefficient, measured in (absorbance at 481 nm)/(OD600).
β	Specific rate of product formation coefficient, measured in (absorbance at 481 nm)/(OD600)/(h).
m/µ	Apparent growth rate of culture, measured in (OD600)/(h).
χ	Dimensionless biomass, the ratio of X2 over X1.
π	Dimensionless product concentration, the ratio of P2 over P1.
Ψs	Dimensionless substrate yield, the ratio of Y_{sx^2} over Y_{sx^1} .
ψ_p	Dimensionless product yield, the ratio of Y_{px^2} over Y_{px^1} .
θ	Dimensionless time, the ratio of time t over the "standard" time before induction td, i.e. the time until strain 2 has reached an OD of 0.5.

Table 4.1 Definitions of parameters and variables in section 4.4 and 4.5.

4.5 Fermentation Model Development and Parameterization

In order to further investigate the performance of the temperature-sensitive lycopene system, a mathematical model describing the process was developed, experimentally validated, and parameterized. The system of monod and luedeking-piret equations presented in the previous section was used to describe the rate of change in biomass, substrate, and product. While many models have been developed to describe the kinetics of microbial fermentation, a combination of monod and luedeking-piret is well-suited for applying to this context, as an extensive body of literature exists verifying their ability to accurately fit data obtained through batch fermentation with E coli, and in particular the context examined here, where a single media component is the limiting substrate, and the synthesis of a single product is of interest to the process¹⁵²⁻¹⁵⁴. These equations, restated from section 4.4, are:

(9)
$$\frac{\mathrm{d}X_2}{\mathrm{d}t} = \frac{\mu_{\max} \cdot S_2 \cdot X_2}{K_{\mathrm{S}} + S_2}$$

(10)
$$\frac{\mathrm{d}S_2}{\mathrm{d}t} = -\frac{Y_{\mathrm{S}/\mathrm{X}_2} \cdot \mu_{\mathrm{max}} \cdot S_2 \cdot X_2}{K_{\mathrm{S}} + S_2}$$

(12)
$$\frac{\mathrm{d}S_2}{\mathrm{d}t} = -Y_{\mathrm{S}/\mathrm{X}_2} \cdot m - Y_{\mathrm{S}/\mathrm{P}_2} \cdot \alpha \cdot m - Y_{\mathrm{S}/\mathrm{P}_2} \cdot \beta \cdot X_2$$

(13)
$$\frac{\mathrm{d}P_2}{\mathrm{d}t} = \mathbf{\alpha} \cdot \mathbf{m} + \boldsymbol{\beta} \cdot \boldsymbol{X}_2$$

Where, as previously, X represents biomass in optical density at 600 nm (OD 600), S represents substrate concentration, in g/L glucose, μ_{max} represents the maximum growth rate in h^{-1} , K_s is the substrate affinity constant, in g/L, and Y_{sx}, Y_{sp}, are the yield coefficients of substrate to biomass, substrate to product, respectively. P represents lycopene concentration. Because in this work lycopene was measured only indirectly, the value of P is absorbance at 481 nm, not mass or concentration. Similarly, it should be noted that Y_{sp} is a conversion between measurements of absorbance and concentration, and as such is not dimensionless (Table 4.1).

To investigate the fermentation kinetics of the temperature-sensitive lycopene synthesis strain, two of the previously developed strains were cultivated, pRNAP-TS (Strain TS) and pRNAP-Dead (Strain D), because for the purposes of validating the model, a comparison between only these two strains provides all the necessary and relevant data. Several other differences from the culturing experiments performed previously needed to be made as well. In particular, a minimal media supplemented with 10g/L of glucose had to be used, as a media with a single limiting carbon source must be used in order to obtain a value for S through direct measurement. The media was also supplemented with 25 mM of ammonium chloride, as this concentration has been shown to be high enough that its gradual depletion will have a minimal impact on apparent growth kinetics over the period investigated, but not so high that any significant negative affect would be seen¹⁵⁵. LB media, though used in previous culturing experiments in this work, was inappropriate here, as far from supporting growth with a single carbon source, the substrate provided by LB is a mixture of numerous amino acids, peptides, and small organic molecules, and numerous examples in the literature show that it remains unclear

exactly which molecules are used, which are used preferentially, or even what fraction of the organic molecules provided by LB are available for use by E. coli at all¹⁵⁶.

Four discrete culturing experiments were performed to gather data for the parameterization of this model: pRNAP-TS at 37 °C (experiment 1, figure 4.13), pRNAP-Dead at 37 °C (experiment 2, figure 4.14), pRNAP-TS at 18 °C (experiment 3, figure 4.15), and pRNAP-Dead at 18 °C (experiment 4, figure 4.16). Measurements were taken every 20 minutes, and each measurement was taken with at most 9 replicates.

The 3 parameters measured directly were X, S, and P. The direct measurement taken for cell concentration was OD 600, as was done previously in this work. S was determined by HPLC analysis on clarified spent culture. P is the measure of lycopene absorbance at 481 nm, using the same method of preparation and extraction described earlier in this work.

The canonical method for determining μ_{max} is to perform a batch fermentation where S >> K_s. Under these conditions, the apparent growth rate μ approaches the value of μ_{max} , so simply measuring μ during the linear growth regime of the fermentation allows the determination of μ_{max} . 6 hour batch cultures with 25 g/L glucose were performed, and the values of μ_{max} in each of the 4 experimental contexts are included in table 4.2. As expected, both strains have a virtually identical μ_{max} at 37 degrees, and a significant decline in this value is seen for both strains at the lower temperature. The decrease relative to the dead intein strain seen in the temperature sensitive strain at 18 degrees is presumably a consequence of the metabolic drain placed on the cell by the expression of the carotenoid synthesis pathway induced by the temperature shift.

Determination of Y_{sx} for experiments 1, 2, and 3 is made possible by the measurements of X and S taken over the course of the fermentations. As is clear from equation 10, dS/dt is linearly proportional to dX/dt by Y_{sx} . Because all boundary conditions are known, this relationship can be integrated with respect to time, and therefore find an analytical solution for Y_{sx} . While there is

some disagreement in the literature as to what impact fermentation temperature should have on E *coli* biomass yield, studies investigating this parameter using heterologous expression strains have, as seen in this work, typically shown a non-trivial reduction in biomass yield coefficient at lower temperatures¹⁵⁷.

Establishing a value for the substrate affinity constant, K_s, has often been a source of difficulty when trying to develop a fermentation model. Efforts to do so typically involve performing a continuous fermentation with the strain of interest in a chemostat and observing the effect of varying substrate concentration on apparent growth rate. However, even this method has resulted in widely varying values of K_s throughout the literature, as numerous factors can affect this measurement, such as non-limiting or trace components in the media, growth phase of the inoculum used for the fermentation, and even the length of the chemostat run. In addition, when direct comparisons are made, values of K_s determined through continuous fermentation have been shown to deviate from those calculated from batch fermentation data by as much as two orders of magnitude, suggesting this method is inappropriate to reasonably estimate K_s for this work. Instead, because values of X and S were measured semi-continuously over the course of the fermentations, enough data is available to solve the system of equations 1 and 2 and find a value of K_s by applying regression analysis and fitting it to the data (Table 4.2). While the high degree of similarity between pRNAP-Dead and pRNAP-TS is to be expected, a surprising result derived from this data is that, even with a temperature drop as large as 19 degrees, only a modest reduction in K_s is observed. However, a survey of the literature reveals that, unlike some other fermentation model parameters, the impact of temperature on *E coli* K_s is unclear, with even large drops in temperature resulting in changes ranging from negligible, to dramatic¹⁵⁸⁻¹⁶¹.

To define parameter values for strain pRNAP-TS at 18 °C the system composed of equations 1, 3 and 4, was solved using the data for X, S, and P obtained from experiment 4, and

informed by parameter values obtained from experiments 1, 2, and 3. It is immediately apparent upon examination of figure 4.16, and its comparison to figure 4.15 that, as demonstrated previously in this work, the expression of the lycopene gene cassette has a fairly large effect on growth rate of the host strain. If it is assumed that the difference in substrate consumption seen is entirely due to the demands of lycopene metabolism and synthesis, it can be calculated that a total of approximately 2.89 g/L of substrate have gone to this purpose. If the maximum theoretical stochiometric yield of lycopene on glucose of 0.1g/g, and the more typical yield reported for lycopene-producing E coli which have not had further pathway engineering, 0.001g/g, are applied to this value, the corresponding values for final titer of lycopene are 0.289g/L are and 2.89mg/L respectively^{162,163}. While these may seem very low, they are actually expected, and agree well with previously reported values of lycopene-producing systems, especially if it is considered that no optimization of the fermentation process was done to improve this value. Even efforts to improve titer through extensive pathway and host engineering, and long-term fed-batch culturing strategies have never resulted in higher final titers than 3.52 g/L in E coli, and values of around 10-90 of mg/L have been reported for systems more similar to that demonstrated here, though even after some host engineering and bioprocess optimization.^{114,164,165}.

If the assumption that Y_{sx} for pRNAP-TS is equal to that already established for pRNAP-Dead is maintained, this data can also be used to estimate the value of Y_{sp} , the relation between absorbance at 481 nm and consumed substrate.

To determine α and β in equation 13, the regression method was used to find values of α and B that fit the data on X and P. Similarly to Y_{sp}, these values relate OD600 to absorbance associated with the formation of product, not mass of product. The somewhat larger value of β compared to α suggests that the formation of product has a relatively weak growth association, and as previously discussed, is presumably exaggerated by the low temperature.
To determine the value of K_s for experiment 4, the same regression analysis was applied as previously, informed by the data obtained for X and S. It could be argued that this value is no longer strictly K_s , as it is often considered to be a measure of the affinity of the cell for its substrate, and the physiological mechanism behind the increased sensitivity to a decline in glucose seen is very likely due to physiological changes due to the stresses associated with lycopene gene cassette expression. In cases where growth is limited by the rate of substrate uptake this definition may be accurate, but Monod himself has stressed that this relationship is only incidental, and that the technical definition of K_s is where it is equal to the concentration of substrate at which the apparent growth rate is equal to half the maximum growth rate. As such, fitting K_s to this data, regardless of the underlying physical mechanism, is appropriate¹⁶⁶.



Figure 4.13 Substrate consumption and biomass accumulation of the pRNAP-Dead strain at 37 degrees. The strain coexpresses both the pRNAP-Dead and the pLYC plasmids. The orange data set represents the concentration of glucose at the indicated time, while the blue represents biomass in terms of OD600. The error bars represent a 95% confidence interval.



Figure 4.14 Substrate consumption and biomass accumulation of the pRNAP-TS strain at 37 degrees. The strain coexpresses both the pRNAP-TS and the pLYC plasmids. The orange data set represents the concentration of glucose at the indicated time, while the blue represents biomass in terms of OD600. The error bars represent a 95% confidence interval.



Figure 4.15 Substrate consumption and biomass accumulation of the pRNAP-Dead strain at 18 degrees. The strain coexpresses both the pRNAP-Dead and the pLYC plasmids. The orange data set represents the concentration of glucose at the indicated time, while the blue represents biomass in terms of OD600. The error bars represent a 95% confidence interval.



Figure 4.16 Substrate consumption, biomass accumulation, and product formation of the pRNAP-TS strain at 18 degrees. The strain coexpresses both the pRNAP-TS and the pLYC plasmids. The orange data set represents the concentration of glucose at the indicated time, the blue represents biomass in terms of OD600, and the gray line represents product concentration in terms of absorbance at 481 nm. The error bars represent a 95% confidence interval.

	pRNAP-Dead at 37 °C	pRNAP-TS at 37 °C	pRNAP-Dead at 18 °C	pRNAP-TS at 18 °C
	(experiment 1)	(experiment 2)	(experiment 3)	(experiment 4)
μ_{max}	0.89±0.0811	0.87±0.0721	0.24±0.0112	0.18±0.00752
Ks	0.128±0.00774	0.131±0.00830	0.113±0.00523	0.296±0.00736
Y _{sx}	2.27±0.0868	2.21±0.0902	2.55±0.0724	2.55±0.0724
Y_{sp}	0	0	0	3.44±0.134
α	0	0	0	(6.98±2.68)*10 ⁻⁵
β	0	0	0	(6.52±5.45)*10-4

Table 4.2 Parameter values of pRNAP-TS and pRNAP-Dead at 37 °C and 18 °C with 95% confidence intervals.

5. Conclusions and Future Directions

Realizing the full potential of metabolic engineering and synthetic biology requires not only the successful expression of novel heterologous enzymes, and the efficient redirection of flux through the desired metabolic pathways, but a thorough understanding and appreciation of the bioprocess context in which such an engineered strain would be used. A strain designed only to direct metabolic flux towards production of some compound of interest to the engineer may result in impressive yields with respect to substrate usage and biomass production, or may achieve previously unseen levels of final titer, but is nonetheless likely to have such low productivity overall that it is unsuitable for implementation as part of a real industrial process. Such a strain, though designed to efficiently synthesize the desired product, uses metabolic resources that would otherwise have gone towards cellular maintenance and biomass accumulation, and therefore slowing production of the physiological infrastructure required generate such compounds in the first place. It is therefore desirable to generate methods by which the strict demarcation of biomass accumulation and product synthesis can be achieved, and by doing so in such a way as to take the economics of a process into consideration, and that utilizes methods providing maximum control over the process to the engineer. Towards this goal, this work has sought to, and succeeded in, implementing a generalizable example of such an externally-induced dynamic metabolic control element.

The element developed for use here employs a T7 RNA polymerase interrupted with the vacuolar membrane ATPase (VMA) intein, derived from S. cerevisiae, which has been mutated to only excise itself from and thereby restore function to the polymerase under conditions of lowered temperature. This has the effect of conferring a temperature-sensitivity phenotype upon

the polymerase, so that by lowering the temperature the culture can be transferred from a biomass accumulation phase to product synthesis. The first sets of experiments were done to verify that the temperature sensitive intein was in fact working as designed, and to use the detection of fluorescence as a way to investigate the performance of the control element with respect to protein, rather than secondary metabolite synthesis. Attempts to investigate the folding and excision of the temperature sensitive intein directly via SDS-PAGE were made, but unsurprisingly were inconclusive, as maintaining the reaction above or below the TS intein's threshold temperatures for excision in vitro proved problematic. However, because the control element is of interest for how it performs within the context of a strain, and controlling expression of a heterologous protein, an indirect method was used to verify its performance. By placing an eGFP gene under a T7 promoter and coexpressing these two plasmids in the same strain, it was shown very clearly that expression was allowed to occur only at the lower temperature at which the culture was grown, 18 degrees celsius. Further investigation of the dynamics of GFP expression over time yielded several other important insights into the control element. Lowering culture temperature amplifies the negative effect that heterologous gene expression, suggesting that this particular control element should be induced at a later stage in growth. Also, a comparison of the GFP expression profiles of the WT T7 RNA polymerase and the TS RNA polymerase-containing strains reveals that no significant difference in lag between induction and fluorescence detection is seen with these strains, suggesting that intein folding is not a rate-limiting step in protein expression with this system. For the next set of experiments a plasmid containing a lycopene expression gene cassette was coexpressed with the experimental polymerases. This again confirmed the result that expression of TS-Intein-controlled genes was only allowed to occur after a temperature down-shift. Just as importantly, this set of experiments showed that unlike most expression systems, the control conferred by the intein is very strict, as

no lycopene was detected before induction in the temperature-sensitive strain, meaning that such tools are valuable not only to improve bioprocess efficiency, but can be used as a solution for the problems associated with expressing toxic proteins in a laboratory setting as well. In this section it is also proven that even without any further modification made to the strain itself, the control conferred by the temperature-sensitive intein causes a notable increase in productivity, as an improvement of approximately 15% is seen in the controlled vs uncontrolled lycopene-expression strains.

Section 4.4 demonstrates a series of simple, generalized mathematical models for use in comparing and predicting the activity of any metabolic control element, and defines several nondimensional variables by which to characterize the performance of these control elements. Direct verification of one of these models found a fairly strong degree of agreement between theoretical and experimental results, but with some notable differences, as the model does not adequately account for the impact of a temperature-downshift on culture growth and gene expression, and the effect of leaky expression on the wild type control strain before the time of induction. In the final section, the impact of the temperature-sensitive intein on the performance of the strain is quantified and used to inform a monod/luedeking-piret fermentation model. While providing few entirely novel insights into the behavior of this system in itself, parameterizing a mathematical model allows the performance of the control element to be discussed in purely mathematical terms, and is a valuable and necessary step towards further optimization and scaling. While this work has succeeded in its aim of implementing and describing an externally induced metabolic control element, several limitations to the particular element used here should be acknowledged.

A temperature-downshift has many advantages in terms of its use in gene expression; the canonical strategy for inclusion body reduction involves lowering temperature, difficulties in heterologous protein can very often be alleviated when cultured at lower temperatures, and, at

least in some cases, the ratio of desired protein to biomass can be improved. Indeed, one of several reasons the particular lycopene gene cassette was chosen to investigate secondary metabolite production in this work is that it has previously been used to demonstrate the value a temperature-shift can have in a culturing strategy, with productivity improving by approximately 20% solely as a result of reducing the temperature from 37 to 20 degrees. However, in many cases a temperature shift is impractical, or even detrimental. At larger fermentation scales lowering the culture temperature can be prohibitively expensive, and even at bench-scale cultures rapid and homogenous cooling can be difficult to achieve, which in turn creates a "messier" demarcation between the induction and growth phases, reducing the overall performance of the system, as indicated by figure 4.11. The solution, then, is to design a control element that a.) has a more step-wise response than temperature-based system has, and b.) scales more easily with respect to culture volume than temperature. A photoactivatable control element could be used to fulfill such a role, and dozens of cellular structures have been shown to dimerize, dissociate, cleave, and otherwise respond to the presence or absence of visible light. Activation with respect to acoustic waves, or a "sonoactivatable" element, could be satisfactory as well, and some basic attempts to engineer gene expression in response to an acoustic signal have been made. Investigation into microbial fuel cells and microbial electrosynthesis has generated a great deal of understanding of the genetics of the involved microorganisms, and this knowledge could very well be used to implement an "electroactivatable" control element once they are better understood, and the responsiveness of certain bacteria to the presence magnetic fields could be a promising area of development for this purpose as well.

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