Toward an era of utilizing methionine overproducing hosts for recombinant protein production in *Escherichia coli*

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Amino acid sequence variants, especially variants containing non-canonical amino acids such as norleucine and norvaline, are a concern during therapeutic protein production in microbial systems. Substitution of methionine residues with norleucine in recombinant proteins produced in *Escherichia coli* is well known. Continuous feeding of amino acids such as methionine is commonly used in *E. coli* fermentation processes to control incorporation of norleucine in the recombinant protein. There are several disadvantages associated with continuous feeding during a fermentation process. For example, a continuous feed increases the operational complexity and cost of a manufacturing process and results in dilution of culture medium which could result in lower cell densities and product yields. To overcome the limitations of existing approaches to prevent norleucine incorporation during *E. coli* fermentations, a new approach using an engineered host was developed that overproduces methionine in the cell to prevent norleucine incorporation without negatively impacting fermentation process performance and product yields. In this commentary, the results on using methionine overproducing hosts for recombinant protein production in *E. coli* and some "watch outs" when using these hosts for recombinant protein production are discussed.

Introduction

Protein based therapeutics have become a key component in the treatment of life-threatening diseases. To ensure the desired safety and efficacy of a therapeutic, it is important to reduce the formation of undesirable product variants during the manufacturing process. Amino acid sequence variants, which can occur as a result of DNA mutations, errors during transcription, translation, and/or post-translational processing, are one of the well-known product variants formed during manufacturing of recombinant protein therapeutics both in microbial[1,2,3] and mammalian systems.[2,4,5] Sequence variants have received considerable attention in recent years as there has been a tremendous advancement in the analytical techniques to detect these variants even at low levels.[2,3,6] Sequence variants in the final drug product can have a potential impact on biological activity and could possibly result in immunogenicity when the product is in injected into patients. As a result of these undesirable substitutions, the product may require intensive analytical characterization, which could result in delays in product approvals by the regulatory health authorities. It is, therefore, important to develop and implement robust methods to prevent these undesirable variants during recombinant protein production.

Sequence variants arising as a result of norleucine incorporation in proteins produced in *E. coli* have been known for over 50 y[7] Norleucine is an unnatural amino acid synthesized as a byproduct in the branched chain amino acid metabolism in *E. coli*.[1] It is a structural analog of methionine and can substitute for methionine residues in proteins because methionyl-tRNA synthetase (MetRS) can use norleucine as a substrate, albeit at a lower efficiency when compared to methionine, to charge the methionyl-tRNA during the translation process.[8,9] Several methods

have been developed to prevent norleucine incorporation in recombinant proteins produced in *E. coli* namely: 1) altering codons in the DNA sequence coding for recombinant protein to remove methionine residues[10]; 2) expressing norleucine degrading enzymes[11]; 3) deleting the genes involved in biosynthesis of norleucine[1]; 4) supplementation of trace elements such as molybdenum, nickel and selenium, in the fermentation medium[12]; and 5) bolus addition or continuous feeding methionine or leucine during the fermentation.[1] However most of these methods suffer from several limitations (discussed in the next paragraph) and are not ideal for the biotechnology industry.

Monoclonal antibodies, especially the IgG class, are rapidly growing class of human therapeutics in many disease areas including oncology, auto immune and infectious diseases.[13] There are 2 highly conserved methionine residues in F_c region of human IgG1 that are important for binding to neonatal F_c receptor (FcRn) and as a result the serum half-life of IgG1.[14-17] Altering methionine codons could potentially decrease serum half-life and hence, this approach may not be applicable for the production of F_c-containing antibodies in *E. coli*. Co-expressing a norleucine degrading enzyme (e.g. glutamate, leucine or valine dehydrogeneases) to prevent norleucine incorporation could reduce product yields due to co-expression. Supplementation of trace elements such as molybdenum, nickel and selenium, reduces norleucine incorporation under oxygen limited conditions, however a majority of *E. coli* fermentations in biotech industry are aerobic and are performed under oxygen excess conditions and hence this method may not be widely applicable. Deleting leucine biosynthetic genes and/or transaminases (genes involved in norleucine biosynthesis in the cell[1]) requires supplementation of high levels of leucine and other branched chain amino acids via continuous or bolus additions to the culture medium. Continuous or bolus additions of methionine during the fermentation process are commonly used methods to prevent norleucine incorporation in *E. coli*. Methionine supplementation reduces the likelihood of methionyl-tRNA mischarging with

norleucine by MetRS during the translation process. However, continuous feeding of a nutrient during a fermentation process has several disadvantages: 1) an additional feed increases the operational complexity and cost (i.e. extra tankage, automation, preparation, sterilization, cleaning, etc.) of the manufacturing process; 2) feed rate and/or feed initiation timing (typically a few hours before product synthesis) are potential critical process parameters due to their impact on norleucine incorporation. Incorrect execution of the methionine feed (e.g., delay in feed initiation, flow control valve issues, transfer line leaks, etc.) during a manufacturing process requires additional discrepancy resolution overhead resulting in delays to product release; 3) continuous feeding or bolus additions during the fermentation process could impact cell densities and possibly product yields due to dilution of the culture medium; 4) an additional feed results in 2 additional parameters, feed rate and feed initiation timing, for evaluation during process characterization studies, which is a key step in the implementation of Quality by Design (QbD) for a biotech product.

Recently, a new approach was developed which overcomes the limitations with the existing approaches to prevent norleucine incorporation during recombinant protein production in *E.coli*.[18] An *E. coli* production host (60E4) was engineered to overproduce methionine by targeting the genes involved in methionine biosynthesis and regulation. Several hosts were constructed in the 60E4 host background containing mutations in the genes: *metA* (encodes for homoserine

O-succinyltransferase which catalyzes conversion of homoserine to *O*-succinyl homoserine, the first unique step in the *de novo* methionine biosynthetic pathway[19]), and/or *metK* (encodes for methionyl adenosyltransferase, which catalyzes the formation of *S*-adenosyl methionine from methionine[20]) or *metJ* (encodes for transcriptional repressor of genes involved in biosynthesis and transport of methionine[21]) (**Fig. 1**). Comparable fermentation performance and product yields were obtained using methionine overproducing production hosts containing a *metA (Y294C)* allele (*metA(Y294C)* encodes for MetA protein with a Y294C mutation) that results in a MetA enzyme feedback resistant to methionine in 3 different recombinant protein production processes. As expected, the *metA(Y294C)* host accumulated high levels of intracellular (150–200 μM) and extracellular methionine (400 μM) during the recombinant protein production phase of the fermentation and hence prevented norleucine incorporation in the recombinant protein. The *metA(Y294C)* allele was introduced into 2 other production hosts, and the *metA(Y294C)* host fermentations showed comparable fermentation performance and product yields as their parent host fermentations in these 2 processes and the product obtained from *metA(Y294C)* host fermentations did not show norleucine incorporation.[18] These results demonstrate the strength of a genetic engineering approach as it overcomes the limitations of existing approaches (e.g. lower costs, simpler processes, no dilution-related effects on yield, and no additional parameters to study during a process
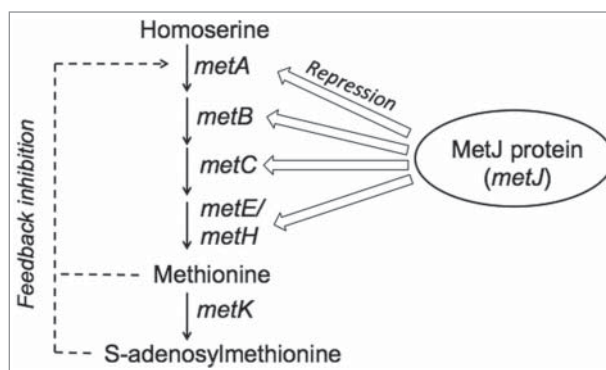

Figure 1. Methionine biosynthesis and regulation in *E. coli*.

characterization exercise, etc.) in preventing norleucine incorporation during *E. coli* fermentations.

Fermentation process conditions and medium composition play an important role in the biosynthesis of methionine in the cell.[22] For example, an elevated temperature could result in aggregation and proteolysis of MetA, or a pH that is suboptimal for catalytic activity of an enzyme in the methionine biosynthetic pathway, could affect methionine biosynthesis.[23,24] Similarly, dissolved oxygen and glucose concentrations in the medium affect methionine biosynthesis.[25,26] Fermentation process conditions that affect methionine biosynthesis in the cell could potentially lead to norleucine incorporation in the recombinant protein when using a methionine overproducing host for recombinant protein production. Alternatively, process conditions that lead to increased biosynthesis of methionine could potentially have an impact on product yields due to a competition for glucose, which is a carbon and energy source in the cell, between pathways that lead to methionine overproduction and product synthesis. For demonstrating overall robustness of methionine overproducing hosts in preventing norleucine incorporation, a bolus of norleucine was added (final concentration of 0.15 mM in the culture medium) during the fermentation process using a *metA(Y294C)* host a few hours before synthesis of the recombinant protein.[18] Levels of norleucine in the recombinant protein were <0.5%, suggesting that the host makes sufficient methionine to control the extent of methionyl tRNA mischarging with norleucine to very low levels even under these conditions. It may be worthwhile to evaluate the *metA(Y294C)* allele containing production hosts under a range of process conditions typically performed during process development and characterization studies and analyze for product yields and norleucine in the recombinant protein before utilizing these hosts for biologic manufacturing processes. Studies along these lines are on-going in our laboratory.

Incorporating a heterologous methionyl-tRNA synthetase or an engineered native MetRS, which cannot charge the methionyl tRNA with

norleucine, into the *E. coli* chromosome could be a powerful approach to eliminate norleucine incorporation. While there is not much evidence on the existence of a heterologous methionyl-tRNA synthetase enzyme with no activity toward norleucine, protein engineers over the last couple of decades have developed and established methods to engineer tRNA synthetases for incorporation of a wide variety of non-canonical amino acids into proteins for studying protein structure and function and adding unique functionality to proteins.[27-29] The *E. coli* MetRS enzyme has been engineered to efficiently incorporate a norleucine analog, azido norleucine, into proteins thus providing structural insights into the residues that are important for binding of azido norleucine in the active site of MetRS.[30] Given that the crystal structures of *E. coli* MetRS with[31] and without[32] bound methionine and several methionine analogs[33] are solved and residues that are critical for binding of azido norleucine is known,[30] it should be possible to exploit this wealth of structural data and engineer the enzyme's active site to decrease or even eliminate its activity toward norleucine.

In addition to norleucine substitutions, multiple other sequence variants have been observed at relatively low levels (<0.2%) in the recombinant proteins purified from the *metA(Y294C)* host fermentations. However, similar levels of sequence variants were also observed in the wild-type *metA⁺* host fermentations which used continuous methionine feeding indicating that these sequence variants are unrelated to methionine overproduction in the cell. These low level sequence variants are consistent with recent studies which demonstrated that many low level sequence variants exist in both recombinant and endogenous proteins produced in microbial systems.[2,3]

In summary, we conclude that utilizing methionine overproducing hosts for manufacturing recombinant proteins in *E. coli* is a promising approach to prevent norleucine incorporation and will lead to simpler and more cost effective manufacturing processes. Demonstrating process robustness with methionine overproducing hosts under a range of process conditions will help usher the microbial

fermentation community toward a new era of using methionine overproducing hosts for *E. coli* recombinant protein production processes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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