MIT MAHE X IISER PUNE 2 PARTNERSHIP

Protein Production

IISER Pune II used prokaryotic *Escherichia coli* as the chassis for expressing the proteins, i.e., the antibodies. However, while looking at the different avenues and chassis for protein expression, they had the opportunity to explore many other such systems which can be tried out to maximize and optimize protein production.

For the purpose of peptide purification, MIT_MAHE designed a vector system where the His tag was attached to the antimicrobial peptide along with a self-cleaving intein. Nevertheless, while examining the different methods for peptide purification, they found out that there are many other such processes that can be used to enhance and optimize peptide purity.

Protein expression and purification are two of the most significant steps in protein production. In the process of our partnership, the teams collated this guide to help other iGEM teams carry out these recombinant protein expression and purification processes.

EXPRESSION

I. INTRODUCTION

Recombinant protein expression has a rich history, and technology has progressed to the point of allowing us to choose any of multiple expression systems to express proteins of interest, from bacteria to yeast to mammalian cell lines. Each system offers unique advantages and disadvantages, which must be analysed before a choice is made. Some of the more common choices are listed and analysed below.

II. EXPRESSION CHASSIS

A. Bacteria

Bacterial production is the most elementary of all these systems. Culturing can be done very cheaply, both in the lab and in larger industrial settings. It is most helpful for simple, monomeric proteins that do not have complex structures or disulfide bonds.

It does vary from protein-to-protein, requiring significant and sensitive optimisation, but it is also much faster than other systems, with a bacterial like *E.coli* not taking more than a day, while culturing CHO cells in suspension, for instance, takes several days.

Bacterial systems often fall short for larger, more complicated proteins, but the advantages above mean that the issues with bacterial production are being actively tackled by scientists all across the world. For example, NEB developed Shuffle *E.coli* a few years ago, a strain with some modified reductive pathways to allow complex proteins like antibodies to fold in its cytoplasm [1].

Another issue with bacterial systems is that they lack the machinery for N-linked glycosylation, and their O-linked glycosylation machinery might not match that of the original host of the protein. So, proteins that depend on these modifications for functionality will not work if produced in bacteria.

There are many choices within bacterial expression for systems as well, with *E.coli* strains like BL21 doing most of the heavy lifting. *B.subtilis* is a gram-positive bacterium, which makes downstream processing a little easier; it is being actively studied to optimise for protein expression. *Lactobacillus* is also being studied for protein expression, but *E.coli* remains the overwhelming workhorse.

B. Mammalian cell lines

These are challenging and slow to culture, requiring more expensive media. Under the right conditions, they can accomplish high yields of very complex proteins, with mammalian post-translational modifications. This makes them invaluable in the production of therapeutic proteins like antibodies [2], which are large, multimeric and need specific kinds of glycosylation to be functional. The biggest players in this field are Chinese Hamster Ovary (CHO) cells.

That being said, alternatives to mammalian cell lines are being investigated for the reasons above, expense and time. We spoke to Dr Mughda Gadgil from the National Chemical Laboratory, who told us that "you go to a CHO cell when you have to go to a CHO cell".

C. Yeast

Yeast cells represent a middle ground between the two above systems - it is a eukaryotic system, but still retains the ease and accessibility of bacterial culture. It is capable of producing quite complex proteins with post-translational modifications, though not identical to that of mammalian cells [3].

One issue with yeast systems is that they tend to show different patterns of glycosylation compared to mammalian cells, which interferes with the functionality as well as the processing of the molecule. This is also being addressed by current research, trying to mimic mammalian glycosylation patterns in yeast.

Many other systems exist for recombinant protein production, though all less developed than the above - insects, algae, cell-free systems and so on [4].

II. ALTERNATIVE VECTORS

Vectors are chosen depending on the expression system, but all vectors typically have -

A. Promoter*s*

Promotoers allow us to adjust the degree of control we have over production of the protein. The strength of the promoter can be changed, as can the extent to which is triggered constitutively (leaky expression) [2]. It can affect the yield and folding of the protein, so it is important to experiment with [5].

B. Selection markers

Selectable markers allows us to differentiate between the cells producing our protein, and those that are not; it is a fundamental element of transforming cells with the vector [6]. It is typically a secondary gene in the vector that gives the cells an easily distinguishable characteristic. Markers also vary across expression systems.

Broadly, they fall under two categories -

1) *Positive selection* - Conferring the cell with a selective advantage, such as the ability to survive in a deadly antibiotic. This is the easier of the two, as normal cells can be eliminated in the first step.
2) *Negative selection* - Conferring the cell with a selective disadvantage, like the inability to survive without a particular compound. This method is more challenging than the first, as the transformed cells must first be grown along with the normal cells, followed by a second phase to check which ones die under normal conditions.

HIS TAG PURIFICATION

I. INTRODUCTION

Affinity tags have become significantly important tools for the expression and purification of recombinant proteins as they have the ability to facilitate the detection of recombinant proteins, increase the efficiency of the purification of their fusion partners along with a positive impact on the production yield of the recombinant proteins. However, the use of affinity tags hinders the preliminary structure and function of the overall fusion system. Therefore, efficient methods for affinity tag removal are required. Recombinant proteins' purification is based on their chemical properties or Immunoaffinity. Based on this, affinity purification procedures have also been formulated to add an affinity tag to these proteins of interest. For example, GST and His tags have enabled us to perform one-step purifying of the recombinant proteins. Enzymatic methods cleavage sites for 3C Protease, Factor Xa, TEV Protease, Thrombin, Caspases, and TAGZyme have been inserted between the affinity tag and the desired polypeptide, which in turn enables proteolytic cleavage and purification on an affinity column like to remove the tag. Chemical methods like the usage of CNBr and novel purification products have also been utilized for affinity tag purification that is attached to a recombinant protein. Although recombinant proteins are purified rapidly using these strategies, the end product contains several extra amino acids, digestive mixtures, and proteolytic enzymes; thus, additional purification steps such as IMAC must also be utilized.

II. ENZYMATIC METHODS

A. Endoproteases

1) TEV protease [7]

➔ *Recognition Site:* ENLYFQG

➔ *Cleavage Site:* ENLYFQ^G (Between Q and G)

TEV is an endoproteolytic reagent that is remarkably specific, easy to produce, and can accept a range of residues at the P1' location of its recognition site, making them apt tools for removing affinity tags. The protease is first synthesized and fused to the C-terminus of MBP, which results in its build-up in a soluble and functional form as opposed to inclusion bodies. The MBP moiety eliminates itself when the fusion protein cleaves itself in vivo, releasing a soluble TEV protease catalytic domain and an N-terminal polyhistidine label. A two-stage process is done to purify the His7-tagged TEV protease. The first stage is Immobilized metal affinity chromatography (IMAC), followed by gel filtration. Despite this method being a suitable approach, it has its limitation. Certain fusion proteins are essentially inadequate substrates for TEV

protease. This is because of the steric blockage if the companion protein's ordered structure or the protease cleavage site is too close or if the fusion protein is present as soluble aggregates. This issue can sometimes be avoided by employing a lot of TEV protease and/or conducting the reaction at a higher temperature (e.g., room temperature). If that does not work, adding extra residues between the target protein's N-terminus and the TEV protease cleavage site is suggested. Polyglycine, polyhistidine, and a FLAG-tag epitope can be utilized to get better results. TEV protease can be used for straightforward pilot tests and extensive protein preparation by a site-specific endoproteolysis method which in vitro cleaves the affinity tags from recombinant proteins

2) **Factor Xa**

➔ *Recognition Site:* Ile — Glu — Gly — Arg ↓ [8]

➔ *Cleavage Site:* IEGR^ [8]

Factor Xa comprises two disulfide-linked polypeptide chains with apparent molecular weights of 17 and 42 kDa, each containing several internal disulfide bonds, rendering the enzyme sensitive to reducing agents. Factor Xa also binds calcium ions and, therefore, should not be used in the presence of chelating agents EGTA and EDTA [9]

3) **Thrombin** [9]

➔ *Recognition Site:* LVPRGS

➔ *Cleavage Site:* LVPR^GS

Thrombin is a disulfide-linked heterodimer with three intramolecular disulfide bonds in one of its two chains, making Thrombin sensitive to reducing agents. The activity efficiency of Thrombin is optimal at temperatures of 45 °C (maximal activity at 15 °C) and a pH range of 5–10.

Thrombin inactivation can be done by adding phenylmethylsulfonyl fluoride (PMSF) or Aminoethyl benzenesulfonyl fluoride hydrochloride (AEBSF).

4) **HRV 3C Protease** [10]

➔ *Recognition Site:* Leu-Glu-Val-Leu-Phe-GlnGly-Pro

➔ *Cleavage Site:* LEVLFQ^GP

For cleavage, a co-fermentation method or a post-fermentation method can be used. The Cofermentation method is when the strains expressing HRV 3C protease and the substrates are mixed before being induced with IPTG, and the post-fermentation method is when they are mixed

after being induced with IPTG. This is followed by cell disruption in then incubated overnight at 4°C. As for the Purification, a cell lysate purification system based on HRV 3C protease (CLP3C method) is followed. The soluble cytoplasmic component was combined with Ni-NTA resin to recover the target protein. Besides the CLP3C technique being simple and inexpensive, it has multiple advantages. Commercial HRV 3C protease is not required.

Additionally, neither the target protein nor the protease needs to be purified. With this method, less degradation of the target protein takes place. Another pro of this method is that since the cleavage reaction takes place in a crude cell lysate, a reaction buffer is not required. The HRV 3C protease does not need cofactors or metal activators to function in a range of buffers. The intense activity of HRV 3C protease at low temperatures is another characteristic that sets it apart from other endoproteases. Among the aforementioned proteases, it has the maximum activity at 4°C.

Consequently, it is perfect for the low-temperature procedure of purifying unstable proteins. It uses both in-solution and on-column techniques to cleave protein tags. After purifying the target protein, the in-solution approach combines the protease with a suitable buffer. To get rid of HRV 3C protease, the second round of purification using IMAC or glutathione resin is done after an overnight incubation period. The target protein is combined with HRV 3C reaction buffer when using the on-column technique, and it is subsequently bound to an iMac or glutathione column. The target protein is then released into the flow-through by removing the protein tag from the resin, which is done by resuspending it in HRV 3C reaction buffer enriched with the desired amount of protease. The resin still has protease on it. Due to its distinctive selectivity and strong activity at low temperatures, HRV 3C protease is frequently utilized as a tool to remove fusion tags from recombinant proteins in gene engineering.
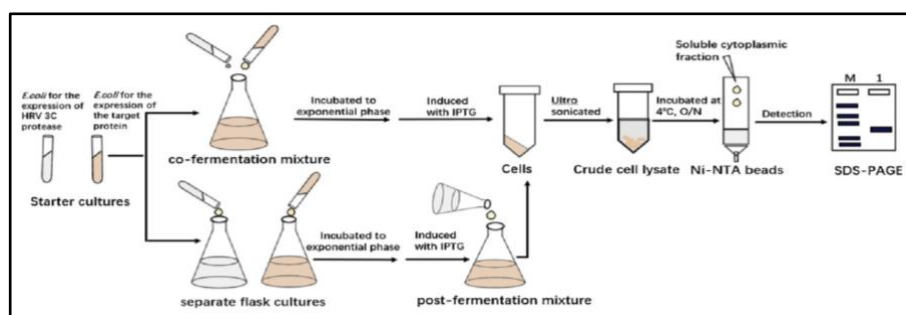

Figure 1. Cell lysate purification using HRV 3C Protease [4]

5) Caspase

Caspases (Cysteine-dependent aspartate-specific proteases) are enzymes having defined substrate specificity. Caspases have a cleavage site that leaves residual amino acids at the N-terminus of the recombinant protein. Several caspases, like Caspase-2, Caspase-3, and Caspase-6, are used to remove affinity tags.

1) *Caspase-2* [11]*:* Caspase-2 is unique among other caspases in its ability to recognize a specific pentapeptide instead of just a tetrapeptide with high affinity. Immediately after the P1 amino acid of the recognition sequence is where the cleavage occurs. VDVAD is the preferred cleavage site for caspase 2.

2) *Caspase-3* [12]*:* The caspase-3 protease system provides an opportunity to procure all the benefits provided by N-terminal fusion proteins while deleting unwanted amino acids on the N-terminus of the protein of interest. Caspase-3 is highly active and cleaves with high specificity C-terminal to DXXD motifs found in the flexible protein regions, leaving no residual amino acids on the N-terminus of the target protein.

3) *Caspase-6* [13]*:* The working of Caspase-6 on its cleavage site has more efficiency and specificity when compared to the factor Xa or the thrombin cleavage site. Caspase-6 has a cleavage site at VEMD. However, VEID also works equally well.

Noting that the primary recognition sites for all caspases consist of four amino acids followed by aspartate at the last (P1) position.

B. Exopeptidases

1) TAGZyme

TAGZyme is an engineered aminopeptidase that does not affect the native protein sequence during tag removal. TAGZyme is an ideal enzymatic system for efficiently removing N-terminal His-tags. DAPase is a recombinant dipeptidyl peptidase I, which is a significant part of the TAGZyme, having the ability to cleave dipeptides sequentially from the N-terminus of any protein. The TAGZyme is only applicable if the amino acid sequence does not contain an Arginine, lysine, or proline at the N terminus. TAGZyme has an efficiency of >95 % for His Tag removal and can be utilized with short treatment periods of around 1 h using low Concentrations of the enzyme at low temperatures, in turn preventing protein denaturation [14]. The DAPase enzyme digestion is stopped when the enzyme comes to a "DAPase stop point," which is an amino acid motif that cannot serve as a substrate [15].

Table 1. DAPase stop points [15]

Amino Acid	DAPase stop point (↓) sequence*
Lysine (Lys, K)	Xaa-Xaa...Xaa-Xaa ↓ Lys-Xaa...
Arginine (Arg, R)	Xaa-Xaa...Xaa-Xaa ↓ Arg-Xaa...
Proline (Pro, P)	Xaa-Xaa...Xaa-Xaa ↓ Xaa-Xaa-Pro-Xaa...

Glutamine (Gln, Q)	Xaa-Xaa...Xaa-Xaa ↓ Gln-Xaa...
Isoleucine (Ile, I)	Xaa-Xaa...Xaa-Xaa ↓ Xaa-Ile-Xaa-Xaa...

Suppose our recombinant protein does not contain a DAPase stop point. In that case, we can insert a glutamine codon into the expression vector where the glutamine in the expressed protein is converted to pyroglutamate, a stop point for the DAPase enzyme. This conversion is done by certain treatments in a subtractive immobilized Metal Affinity Chromatography (IMAC) column [15]. Here:

1) *DAPase enzyme digestion:* Cleaved using glutamine acyltransferase (Qcyclase). Qcyclase converts the glutamine residue in the N-terminal to pyroglutamate [14].

2) *pGAPase Treatment:* Removes the N-terminal pyroglutamyl residue leaving only the purified tag-free protein in the native N-terminus [14].
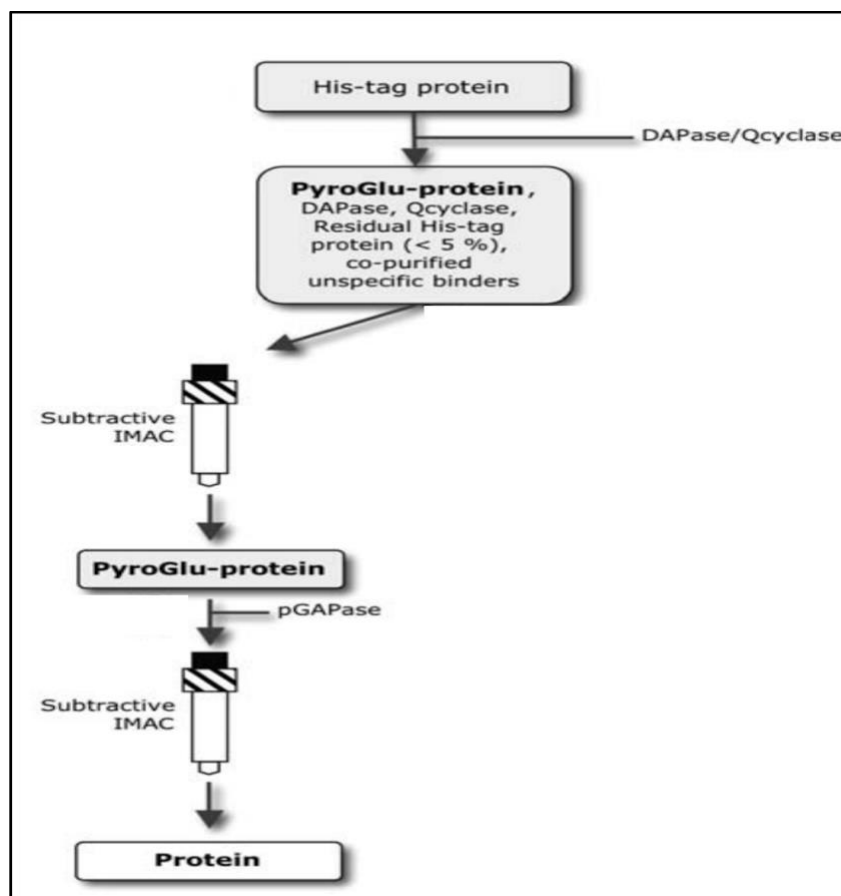

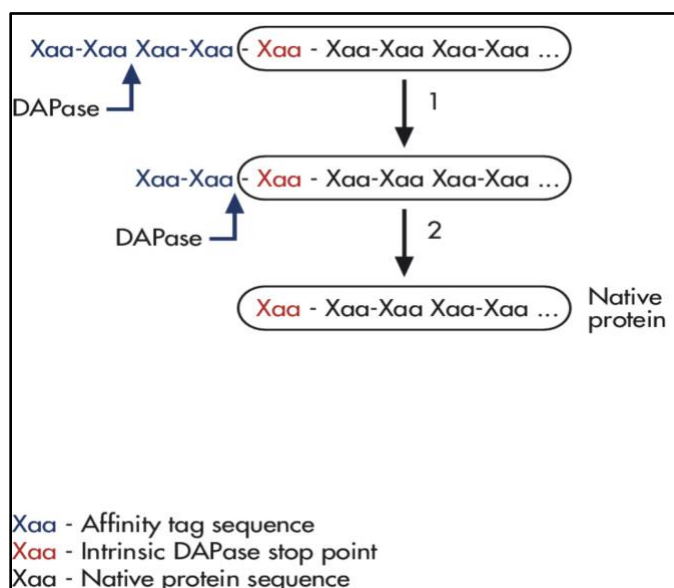Figure 2. TAGzyme cleavage scheme of recombinant protein without DAPase stop point [14]

Figure 3. TAGzyme cleavage scheme of recombinant protein with DAPase stop point [15]

III. CHEMICAL METHODS

A. Cyanogen bromide (CNBr) [16]

➜ Cleavage at a single methionine between the His tag and the peptide

This method can be applied to all recombinant protein production systems, from viruses to eukaryotic expression systems. Under certain conditions, Cyanogen bromide (CNBr) has been known to cleave polypeptides, specifically at the methionine residue. The basic flow of purification:

1) Immobilized metal ion-affinity chromatography is used for purification, with a histidine tag causing the retention of target molecules on IMAC columns.

2) The tag is then cleaved with CNBr at the methionine placed between the tag and the polypeptide.

3) *Purification by IMAC*

   a) By using IMAC, the fusion protein can be purified in a single step by containing an initiation of translation sequence and a histidine tag.

   b) A Ni-NTA agarose resin was used to load bacterial cell lysates.

   c) The fusion protein was eluted after washing with increasing imidazole concentrations and urea.

    d) The material was analyzed using SDS-PAGE combined with Coomassie blue staining and silver nitrate staining.

4) *CNBr Cleavage*

    a) A single methionine residue was inserted next to the peptide coding region to cleave only the protein of interest (mainly for long synthetic peptides)

    b) Using 20- to 100-fold molar excess of CNBr produces a high protein yield compared to other chemical cleavage methods.

    c) To analyze the product, chromatography and mass spectrometry were done after a second IMAC purification, then emoved the histidine tag.

It is also possible to cleave Trp/X using glacial acetic acid and 9 M HC1 in the presence of DMSO and CNBr in addition to Met/X cleavage. Instead of cleaving methionine, methionine sulfoxide is formed.

IV. OTHER METHODS

A. Affinity agarose resins [17]

➔ *Example:* Glutathione, cobalt chelate

Protein-interaction targets can be captured by a "bait" protein using an affinity resin rather than an antibody. This is known as a "pull-down assay." The bait protein may be tagged by a glutathione S-transferase (GST) or 6× histidines (His), and the tagged bait proteins specifically bind to glutathione or cobalt chelate agarose resin.

B. Bio-Scale™ Mini Profinity™ IMAC Cartridges [18]

Bio-Scale Mini Profinity IMAC kit cartridges are mini cartridges filled with Profinity Ni-charged IMAC resin. They purify recombinant proteins containing histidine tags by using the principle of immobilized metal affinity chromatography (IMAC). This technique relies on the His-tag's affinity for immobilized transition metals. The Profinity IMAC resins provided in the kit is the UNOsphere™ beads, which contain iminodiacetic acid (IDA) as the chelating ligand for divalent and trivalent metal ions. The chemical structure of these beads allows highly selective or specific binding of recombinant His-tagged proteins when charged with transition metals such as $Ni2+$, $Zn2+$ and $Cu2+$. Features of Profinity IMAC resins include:

1) It can be utilized in high flow rates, non-denaturing conditions, and denaturing conditions

2) Provides optimal ligand density for higher purity of target protein

3) High mechanical strength

4) Stable from pH 1 to 14

5) No effect by denaturing agents, reducing agents, or detergents.

The cartridges have a double wall that provides extra durability and allows smoother, reliable runs at pressures up to 45 psi pressure with the aqueous buffers of recombinant protein separation. These cartridges can be used on a peristaltic pump, or any chromatography system. The cartridges can then be used with a Luer-Lok syringe for the elution step. Other features include:

1) Simple fittings for easy connection to any system, including fittings for connection to FPLC and HPLC systems

2) Chemically compatible polypropylene parts

3) A wide range of chromatography media

C. Self-Cleaving Chitin-Binding Affinity Tag [19]

The chitin-binding domain (CBD) tag can be utilized with the ΔI-CM intein to create a self-cleaving purification tag. The gene of interest is fused with the tag, creating a fusion protein that can be purified using chitin resin. This makes it more efficient to get the protein you need.

Recombinant proteins expressed in E. coli can be readily purified using the intein self-cleavage system. This system requires a pH and temperature shift to induce self-cleavage, which liberates the free target protein. This approach can be used to purify any recombinant protein that can be expressed in E. coli and has the potential to be applied to a wide variety of additional tags and expression hosts.

D. Inteins [20]

A recombinant protein can be separated from its affinity tag during affinity purification using inteins' capacity to catalyze a regulated peptide bond cleavage process. Inteins and a chitin-binding domain have been coupled to create a self-cleaving affinity tag that enables extremely selective capture of the fusion protein on a low-cost substrate. Reduced cell density cultures have successfully used this purification method on a lab scale. Thiol or pH and temperature-initiated in vitro cleavage reactions could occur with intein fusion proteins generated under high cell density fermentations, and the stability of the protein is seen in vivo after induction for a considerable period of time. As a result, it is possible to produce recombinant proteins on an industrial scale using a protein purification technique that employs inteins. Most inteins have a cysteine (Cys) or serine (Ser) residue at the N-terminus, an asparagine (Asn) residue at the C-terminus, and histidine (His) as the penultimate residue, according to intein sequence alignment. There is no other

homology found in the extein sequences. However, most exteins have the first residue of the C-terminal as cysteine, serine, or threonine. The protein splicing processes entail the conserved residues at the intein splice junctions. These protein splicing events can be interfered with in vivo by changing the amino acids in one or more of the intein's catalytic residues. For instance, a thiol reagent, such as dithiothreitol, can cleave the complete fusion protein at the N-terminal of the intein while still preventing splicing in vivo when the C-terminal Asn is replaced by alanine (Ala) (DTT). Whereas by using DTT, a pH shift, or even a temperature change, other alterations can inhibit in vivo cleavage while allowing cleavage at both intein terminals in vitro.

The intein technique not only discards the necessity for protease treatment but also enables the use of a low-cost chromatography substrate to carry out a highly selective capture process. Minimizing self-cleavage during protein expression (in vivo cleavage) and maximizing the cleavage reaction during purification (in vitro cleavage) are some limitations to the protein purification process employing inteins. Other factors that play a significant role in controlling the intein cleavage are the intrinsic splicing activity of the intein and the appropriate folding of the intein and its surrounding domains. Though it has some limitations, the recovery costs of commercial recombinant proteins may be significantly lowered by the intein-mediated purification technique.

Various Intein fusion systems using different inteins have also been created for affinity protein purification using this method.

V. Conclusion

Purification schemes for any protein can be designed by adding the oligo-histidine tag (His-tag) to the protein N- or C-terminus. In the laboratory, immobilized metal affinity chromatography (IMAC) can be used to purify recombinant proteins from crude cellular feedstocks containing poly-histidine fusion tags. The rest of his-tagged proteins, along with their functions and characteristics, are less affected by protein immobilization because only his-tags are involved. Researchers have found that it is possible to isolate His-tagged proteins in one step in many research labs, as removing the tags and achieving extreme purity is not always necessary. Incorporating a fusion tag on the target protein makes it easier to choose a purification method without knowing much about it. It is still challenging to perform efficient two-step chromatographic isolation at a large scale. However, it might be an attractive option in many cases where both the IMAC steps and cleavage of the tags are efficient. As a result of advances in bio-separation, more cost-effective methods are needed to meet the most demanding product specifications. The importance of cleavage precision and efficiency must be stressed again, as they are the critical steps that can affect the overall yield and cost of the process. As a final advantage, the technology can be used to prepare a wide variety of recombinant proteins fused with polyhistidine tags and to capture these proteins in a highly specific manner, making it a generic and highly valuable tool for any analysis that relies on biomarker molecules.

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